PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/29, 15/82

A2

(11) International Publication Number: **WO 96/38560**

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/GB96/01332

(22) International Filing Date: 3 June 1996 (03.06.96)

(30) Priority Data:
9511196.9 2 June 1995 (02.06.95) GB

(71) Applicant (*for all designated States except US*): JOHN INNES CENTRE INNOVATIONS LIMITED [GB/GB]; Norwich Research Park, Colney Lane, Norwich NR4 7UH (GB).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): DEAN, Caroline [GB/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB). MACKNIGHT, Richard, Colin [NZ/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB). BANCROFT, Ian [GB/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB). LISTER, Clare, Katharine [GB/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB).

(74) Agents: WALTON, Seán, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENETIC CONTROL OF FLOWERING

(57) Abstract

FCA genes of *Arabidopsis thaliana* and *Brassica napus* are provided, enabling flowering characteristics, particularly timing of flowering, to be influenced in transgenic plants. Timing of flowering may be delayed or hastened using sense and antisense expression, also various mutants and alleles, including alternatively spliced forms.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

GENETIC CONTROL OF FLOWERING

This invention relates to the genetic control of flowering in plants and the cloning and expression of genes involved therein. More particularly, the invention relates to the cloning and expression of the FCA gene of *Arabidopsis thaliana*, and homologues from other species, and manipulation and use of these genes in plants.

Efficient flowering in plants is important, particularly when the intended product is the flower or the seed produced therefrom. One aspect of this is the timing of flowering: advancing or retarding the onset of flowering can be useful to farmers and seed producers. An understanding of the genetic mechanisms which influence flowering provides a means for altering the flowering characteristics of the target plant. Species for which flowering is important to crop production are numerous, essentially all crops which are grown from seed, with important examples being the cereals, rice and maize, probably the most agronomically important in warmer climatic zones, and wheat, barley, oats and rye in more temperate climates. Important seed products are oil seed rape, sugar beet, maize, sunflower, soybean and sorghum. Many crops which are harvested for their roots are, of course, grown annually from seed and the production of seed of any kind is very dependent upon the ability of the plant to flower, to be pollinated and to set seed. In horticulture, control of the timing of flowering is important. Horticultural plants whose flowering may be

controlled include lettuce, endive and vegetable brassicas including cabbage, broccoli and cauliflower, and carnations and geraniums.

Arabidopsis thaliana is a facultative long-day plant, flowering early under long days and late under short days. Because it has a small, well-characterized genome, is relatively easily transformed and regenerated and has a rapid growing cycle, *Arabidopsis* is an ideal model plant in which to study flowering and its control.

One of the genes required for rapid floral induction is the *FCA* gene (Koornneef et al 1991). Plants carrying mutations of this gene flower much later than wild-type under long photoperiods and short photoperiods. There is a considerable range in flowering time within different mutant fca alleles. The most extreme (fca-1) flowers under long photoperiods with up to 40 leaves whereas *fca*-3, *fca*-4 flower with ~20 rosette leaves compared to 9 for wild-type Landsberg *erecta*). The late flowering of all the *fca* mutants can be overcome to early flowering in both long and short photoperiods if imbibed seeds, or plants of different developmental ages, are given 3-8 weeks at 4°C - a vernalization treatment (Chandler and Dean 1994).

We have cloned and sequenced the *FCA* gene of *Arabidopsis thaliana*, a homologue from *Brassica* and mutant sequences.

According to a first aspect of the present invention there is provided a nucleic acid molecule comprising a

nucleotide sequence encoding a polypeptide with *FCA* function. Those skilled in the art will appreciate that "*FCA* function" refers to the ability to influence the timing of flowering phenotypically like the *FCA* gene of *Arabidopsis thaliana*, especially the ability to complement an *fca* mutation in *Arabidopsis thaliana*.

Nucleic acid according to the invention may encode a polypeptide comprising the amino acid sequence shown in Figure 2, or an allele, variant, derivative or mutant thereof. Particular variants include those wherein the amino acid residues up-stream of the third methionine and/or up-stream of the second methionine in the amino acid sequence of Figure 2 are not included. Variants, mutants and derivatives of nucleic acid encoding such shorter polypeptide are of course provided by various embodiments of the present invention.

Nucleic acid according to the present invention may have the sequence of an *FCA* gene of *Arabidopsis thaliana*, or be a mutant, variant (or derivative) or allele of the sequence provided. Preferred mutants, variants and alleles are those which encode a protein which retains a functional characteristic of the protein encoded by the wild-type gene, especially the ability to promote flowering as discussed herein. Promotion of flowering may advance, hasten or quicken flowering. Other preferred mutants, variants and alleles encode a protein which delays flowering compared to wild-type or a gene with the sequence provided. Changes to a sequence, to

produce a mutant or variant, may be by one or more of insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the insertion, deletion or substitution of one or more amino acids. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included. Particular variants, mutants, alleles and variants are discussed further below.

A preferred nucleic acid sequence covering the region encoding the *FCA* gene is shown in Figure 1 and the predicted amino acid sequence encoding the *FCA* ORF is shown in Figure 2. Nucleic acid may be subject to alteration by way of subsitution of nucleotides and/or a combination of addition, insertion and/or substitution of one or more nucleotides with or without altering the encoded amino acids sequence (by virtue of the degeneracy of the genetic code).

Nucleic acid according to the present invention may comprise an intron, such as an intron shown in Figure 1, for instance intron 3 (as in various embodiments e.g. as illustrated herein), whether or not the encoded amino acid sequence is altered. For example, the variant *FCA* α_B, whose nucleic acid sequence is shown in Figure 3, comprises intron 3 of the sequence of Figure 1, such that translation of the sequence results in a different amino acid sequence from that of Figure 2 (intron 3 of Figure 1 contains a stop codon at 3026-3028 that is potentially used in transcripts).

The present invention also provides a vector which comprises nucleic acid with any one of the provided sequences, preferably a vector from which polypeptide encoded by the nucleic acid sequence can be expressed. The vector is preferably suitable for transformation into a plant cell. The invention further encompasses a host cell transformed with such a vector, especially a plant cell. Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome. There may be more than one heterologous nucleotide sequence per haploid genome. This, for example, enables increased expression of the gene product compared with endogenous levels, as discussed below.

A vector comprising nucleic acid according to the present invention need not include a promoter, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence encoding a polypeptide able to influence flowering, eg in *Arabidopsis thaliana* nucleic acid other than the *FCA* sequence. Nucleic acid

according to the present invention may comprise cDNA, RNA, genomic DNA and may be wholly or partially synthetic. The term "isolate" may encompass all these possibilities.

The present invention also encompasses the expression product of any of the nucleic acid sequences disclosed and methods of making the expression product by expression from encoding nucleic acid therefore under suitable conditions in suitable host cells, e.g. *E. coli* (see Example 7). Those skilled in the art are well able to construct vectors and design protocols for expression and recovery of products of recombinant gene expression. Suitable vectors can be chosen or constructed, containing one or more appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Transformation procedures depend on the host used, but are well known. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

Purified FCA protein, or a fragment, mutant or variant thereof, e.g. produced recombinantly by expression from encoding nucleic acid therefor, may be used to raise antibodies employing techniques which are standard in the art, as exemplified in Example 7. Antibodies and polypeptides comprising antigen-binding fragments of antibodies may be used in identifying homologues from other species as discussed further below.

Methods of producing antibodies include immunising a mammal (eg human, mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82). Antibodies may be polyclonal or monoclonal.

As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, eg using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies raised to a polypeptide or peptide can be used in the identification and/or isolation of homologous polypeptides, and then the encoding genes. Thus, the present invention provides a method of identifying or isolating a polypeptide with FCA function (in accordance with embodiments disclosed herein), comprising screening candidate polypeptides with a polypeptide comprising the antigen-binding domain of an antibody (for example whole antibody or a fragment thereof) which is able to bind an FCA polypeptide or fragment, variant or variant thereof or preferably has binding specificity for such a polypeptide, such as having the amino acid sequence shown in Figure 2 or Figure 8b. Specific binding members such as antibodies and polypeptides comprising antigen binding domains of antibodies that bind and are preferably specific for a FCA polypeptide or mutant, variant or derivative thereof represent further aspects of the present invention, as do their use and methods which employ them.

Candidate polypeptides for screening may for instance be the products of an expression library created using nucleic acid derived from an plant of interest, or may be the product of a purification process from a natural source.

A polypeptide found to bind the antibody may be isolated and then may be subject to amino acid sequencing. Any suitable technique may be used to sequence the polypeptide either wholly or partially (for

instance a fragment of the polypeptide may be sequenced). Amino acid sequence information may be used in obtaining nucleic acid encoding the polypeptide, for instance by designing one or more oligonucleotides (e.g. a degenerate pool of oligonucleotides) for use as probes or primers in hybridisation to candidate nucleic acid, or by searching computer sequence databases, as discussed further below.

The present invention further encompasses a plant comprising a plant cell comprising nucleic acid according to the present invention e.g. as a result of introduction of the nucleic acid into the cell or an ancestor thereof, and selfed or hybrid progeny and any descendent of such a plant, also any part or propagule of such a plant, progeny or descendant, including seed.

The *FCA* gene encodes a large protein (796 amino acids shown in Figure 2) with homology to a class of proteins identified as RNA-binding proteins (Burd and Dreyfuss 1994). These proteins contain 80 amino acid, RNA recognition motifs (RRMs) and have a modular structure- they can contain several RNA binding domains and auxiliary domains rich in amino acids such as glycine, glutamine and proline. The RRM proteins can be divided into subfamilies based on homology within and around the RRM domains. The FCA protein is most homologous to a subfamily of RNA-binding proteins (cluster 1028.16; identified using the BEAUTY database search, Worley et al., 1995) exemplified by the *Drosophila elav* gene (Robinow et al., 1988). Other members of this family

include the *Drosophila* sexlethal protein; the human nervous system proteins HuD, HuC, Hel-N1, and Hel-N2; and the *Xenopus* proteins elrA, elrB, elrC, elrD and etr-1. FCA has two RNA-binding domains while most of the members of *elav* gene family have three RNA-binding domains. The first two RNA-binding domains of *elav* family (and the spacing between the domains) is similar to the RNA-binding domains in the FCA protein. In common with the FCA protein the *elav* has a region with high glutamine content. There is also a 20 amino acid region near the C terminus of the FCA protein which shows strong homology to ORFs from two genes of unknown function from yeast and C. elegans.

The *FCA* transcript is alternatively spliced. Five forms of the transcript are generated in cells. One, herein called *FCA* transcript β is ~ 2kb and represents premature termination and polyadenylation within intron 3. *FCA* α_A and α_B has 19 of 20 introns spliced out but intron 3 (2kb) remaining. *FCA* α_A is the same as α_B except at intron 11 where different 5′ and 3′ exon/intron junctions are used. *FCA* α_A uses the 5′ exon/intron junction at 7055 bp (genomic sequence Fig.1) and 3′ exon/intron junction at 7377 bp. *FCA* α_B uses the 5′ exon/intron junction at 7130 bp (genomic sequence Fig.1) and 3′ exon/intron junction at 7295 bp. *FCA* transcripts γ_A and γ_B both have intron 3 removed and γ_A and γ_B use the same junctions around intron 11 as α_A and α_B,

respectively. Only γ_B encodes both RNA-binding domains and the conserved C-terminal domain (Figure 10).

RNA-binding proteins have been shown to be involved in several facets of post-transcriptional regulation. The RNP motif forms a β sheet RNA binding surface engaging the RNA as an open platform for interaction with either other RNA molecules or other proteins. One of the most well characterized genes encoding an RNP motif-containing protein is the Drosophila *SEX-LETHAL* gene (Bell et al 1988). The *SEX-LETHAL* protein is involved in altering the splicing of its own and other transcripts within the pathway that determines sex in Drosophila. Only the alternatively spliced product gives an active protein. Thus this gene product is responsible for determining and maintaining the female state. Other RNA-binding proteins have been shown to function by localizing specific transcripts in the nucleus or preventing translation of specific transcripts. Six independently isolated *fca* mutants have been described, and we have identified the sequence changes causing a reduction in *FCA* activity in three cases. The *fca*-1 mutation converted a C nucleotide at position 6861 (Figure 1) into a T. Thus a glutamine codon (CAA) is changed into a stop codon (TAA). The *fca*-3 mutation converted a G nucleotide at position 5271 into an A. The effect of this mutation is to alter the 3' splice junction of intron 7 such that a new 3' splice junction is used 28 nucleotides into exon 8. The *fca*-4 mutation is the result of a rearrangement (an inversion

taking the 3' end of the gene 250kb away) with the break-point at position 4570 (within intron 4).

A further aspect of the present invention provides a method of identifying and cloning *FCA* homologues from plant species other than *Arabidopsis thaliana* which method employs a nucleotide sequence derived from that shown in Figure 1. Nucleic acid libraries may be screened using techniques well known to those skilled in the art and homologous sequences thereby identified then tested. The provision of sequence information for the *FCA* gene of *Arabidopsis thaliana* enables the obtention of homologous sequences from *Arabidopsis* and other plant species. In Southern hybridization experiments a probe containing the *FCA* gene of *Arabidopsis thaliana* hybridises to DNA extracted from *Brassica rapa*, *Brassica napus* and *Brassica oleraceae*. In contrast to most *Arabidopsis genes*, which are normally present on the *B. napus* genome in 6 copies, the *FCA* gene is present twice, on only one pair of chromosomes. An *FCA* homologue from *Brassica napus* has been isolated and sequenced and shows 86.1% average nucleotide sequence homology within the exons, 65.8% within introns and 78% identity at the amino acid level (87% similarity). This *Brassica* gene fully complements a mutation in the *Arabidopsis FCA* gene and can thus be considered as a fully functional homologue. Homologues have also been detected by Southern blot analysis from Antirrhinum, tobacco, sugarbeet, tomato, pea, wheat, maize, rice, rye, Lolium and oats.

The *Brassica* FCA homologue whose nucleotide sequence is given in Figure 8a, including the coding sequence, and whose amino acid sequence encoded by the sequence of Figure 8a is shown in Figure 8b, represents and provides further aspects of the present invention in accordance with those disclosed for the *Arabidopsis* FCA gene. For example, mutants, alleles and variants are included, e.g. having at least 80% identity with the sequence of Figure 8b, though high levels of amino acid identity may be limited to functionally significant domains or regions as discussed.

The present invention also extends to nucleic acid encoding an *FCA* homologue obtained using a nucleotide sequence derived from that shown in Figure 1, or the amino acid sequence shown in Figure 2. Preferably, the nucleotide sequence and/or amino acid sequence shares homology with the sequence encoded by the nucleotide sequence of Figure 1, preferably at least about 50%, or at least about 60%, or at least about 70%, or at least about 75%, or at least about 78%, or at least about 80% homology, most preferably at least about 90% homology, from species other than *Arabidopsis thaliana* and the encoded polypeptide shares a phenotype with the *Arabidopsis thaliana FCA* gene, preferably the ability to influence timing of flowering. These may promote or delay flowering compared with *Arabidopsis thaliana FCA* and mutants, variants or alleles may promote or delay

flowering compared with wild-type. "Homology" may be used to refer to identity.

In certain embodiments, an allele, variant, derivative, mutant or homologue of the specific sequence may show little overall homology, say about 20%, or about 25%, or about 30%, or about 35%, or about 40% or about 45%, with the specific sequence. However, in functionally significant domains or regions the amino acid homology may be much higher. Comparison of the amino acid sequences of the FCA polypeptides of the *Arabidopsis thaliana* and *Brassic napus* genes, as in Figure 9, reveals domains and regions with functional significance, i.e. a role in influencing a flowering characteristic of a plant, such as timing of flowering. Deletion mutagenesis, for example, may be used to test the function of a region of the polypeptide and its role in or necessity for influence of flowering timing.

The nucleotide sequence information provided herein, or any part thereof, may be used in a data-base search to find homologous sequences, expression products of which can be tested for ability to influence a flowering characteristic. These may have *FCA* function or the ability to complement a mutant phenotype, which phenotype is delayed flowering, where the delay can be reversed by a vernalization treatment.

Vernalization is well known in the art and appropriate conditions are at the disposal of skilled artisans. Plants may be vernalized at the seed stage,

immediately after sowing. It may be carried out for 8 weeks, in an 8 hour photoperiod (e.g fluorescent light, PAR 9.5mmol $m^{-2}s^{-1}$, R/FR ratio 3.9) at a temperature of 5oC +/-1oC.

In public sequence databases we recently identified several *Arabidopsis* cDNA clone sequences that were obtained in random sequencing programmes and share homology with *FCA* within both the RRM domains and in the C-terminal regions. BLAST and FASTA searches of databases have identified 23 *Arabidopsis* expressed sequence tags (ESTs) identified. These clones have been obtained and used in low stringency hybridization experiments with different regions of the *FCA* gene (central and 3'). Eight clones show good homology to the 3' part of the *FCA* gene, two clones show good homology to the central part and one clone shows good homology to both (42 A 4 - another RNA-binding protein). Similarly, among randomly sequenced rice cDNAs we have identified 10 rice ESTs. These hybridise to *FCA* genomic and cDNA clones under low stringency conditions. Five clones show good hybridization to *FCA*, particularly C1480.

By sequencing homologues, studying their expression patterns and examining the effect of altering their expression, genes carrying out a similar function to *FCA* in regulating flowering time are obtainable. Of course, mutants, variants and alleles of these sequences are included within the scope of the present invention in the

same terms as discussed above for the *Arabidopsis thaliana FCA* gene.

The high level of homology between the FCA genes of *Arabidopsis thaliana* and *Brassica napus*, as disclosed herein, may also be exploited in the identification of further homologues, for example using oligonucleotides (e.g. a degenerate pool) designed on the basis of sequence conservation.

According to a further aspect, the present invention provides a method of identifying or a method of cloning a FCA homologue from a species other than *Arabidopsis thaliana*, the method employing a nucleotide sequence derived from that shown in Figure 1 or that shown in Figure 8a. For instance, such a method may employ an oligonucleotide or oligonucleotides which comprises or comprise a sequence or sequences that are conserved between the sequences of Figures 1 and 8a to search for homologues. Thus, a method of obtaining nucleic acid whose expression is able to influence a flowering characteristic of a plant is provided, comprising hybridisation of an oligonucleotide or a nucleic acid molecule comprising such an oligonucleotide to target/candidate nucleic acid. Target or candidate nucleic acid may, for example, comprise a genomic or cDNA library obtainable from an organism known to contain or suspected of containing such nucleic acid. Successful hybridisation may be identified and target/candidate

nucleic acid isolated for further investigation and/or use.

Hybridisation may involve probing nucleic acid and identifying positive hybridisation under suitably stringent conditions (in accordance with known techniques) and/or use of oligonucleotides as primers in a method of nucleic acid amplification, such as PCR. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

As an alternative to probing, though still employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences may be used in PCR reactions or other methods involving amplification of nucleic acid, using routine procedures. See for instance "PCR protocols; A Guide to Methods and Applications", Eds. Innis *et al*, 1990, Academic Press, New York.

Preferred amino acid sequences suitable for use in the design of probes or PCR primers are sequences conserved (completely, substantially or partly) between at least two FCA polypeptides able to influence a flowering characteristic, such as timing of flowering, e.g. with the amino acid sequences of Figures 2 and 8b herein.

On the basis of amino acid sequence information oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived.

Preferably an oligonucleotide in accordance with the invention, e.g. for use in nucleic acid amplification, has about 10 or fewer codons (e.g. 6, 7 or 8), i.e. is about 30 or fewer nucleotides in length (e.g. 18, 21 or 24).

Assessment of whether or not such a PCR product corresponds to resistance genes may be conducted in various ways. A PCR band from such a reaction might contain a complex mix of products. Individual products may be cloned and each one individually screened. It may be analysed by transformation to assess function on introduction into a plant of interest.

Generally, nucleic acid according to the invention may comprise a nucleotide sequence encoding a polypeptide able to complement a mutant phenotype which is delayed in flowering, where that delay can be corrected by a vernalization treatment. Also the present invention provides nucleic acid comprising a nucleotide sequence which is a mutant or variant of a wild-type gene encoding a polypeptide with ability to influence the timing of flowering, the mutant or variant phenotype being delayed in flowering with the timing of flowering being corrected by vernalization. These are distinguished from the *CO*

gene reported by Putterill et al 1995, Putterill et al 1993 and the *LD* gene reported by Lee et al 1994. *LD* shows similar characteristics to the *FCA* gene in that a mutation in the gene confers late flowering that is corrected by a vernalization treatment, but *LD* requires a second gene product to influence flowering time in the *Arabidopsis thaliana* Landsberg erecta ecotype (Lee et al 1994, Koornneef et al 1994). Thus in many plant species manipulation of the *LD* gene alone may not influence flowering time. The action of *FCA* is opposite in action to that of phytochromeB, in that mutations in *PHYB* (*hy3*) confer early flowering and introduction of an intact *PHYB* gene into *hy3* mutants restores normal flowering time (Wester] et al 1994). *LD* and *CO* are excluded from the ambit of the present invention. *FCA* and mutants, variants and alleles thereof may not complement an *LD* mutation. *LD* and mutants, variants and alleles thereof may not complement an *FCA* mutation.

The FCA amino acid sequence is totally different from those of CO and LD.

The action of *FCA* can also be distinguished from ectopic expression of meristem identity or MADS box genes that alter flowering time (Weigel and Nilsson 1995, Chung et al 1994, Mandel and Yanofsky 1995, Mizukama and Ma 1992). Apart from an early flowering phenotype, ectopic or overexpression of meristem identity or MADS box genes produces many additional perturbations to both the

vegetative and floral phenotype of the plant (eg. short stature, reduced apical dominance, sterile flowers).

Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of nucleotides where different introns have been removed. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome.

Plants which comprise a plant cell according to the invention are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants and any part or propagate thereof.

The invention further provides a method of influencing the flowering characteristics of a plant comprising expression of a heterologous *FCA* gene sequence (or mutant, allele, variant or homologue thereof, as discussed) within cells of the plant. The term "heterologous" indicates that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, ie by human intervention. The gene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. The heterologous gene may replace an endogenous equivalent gene, ie one which normally performs the same or a similar function in

control of flowering, or the inserted sequence may be additional to the endogenous gene. An advantage of introduction of a heterologous gene is the ability to place expression of the gene under the control of a promoter of choice, in order to be able to influence gene expression, and therefore flowering, according to preference. Furthermore, mutants and variants of the wild-type gene, eg with higher or lower activity than wild-type, may be used in place of the endogenous gene.

The principal flowering characteristic which may be altered using the present invention is the timing of flowering. Under-expression of the gene product of the *FCA* gene leads to delayed flowering (as indicated by the *fca* mutant phenotype and Example 3, antisense experiments) that can be overcome to early flowering by a vernalization treatment; over-expression may lead to earlier flowering (Examples 2, 4 and 5). This degree of control is useful to ensure synchronous flowering of male and female parent lines in hybrid production, for example. Another use is to advance or retard the flowering in accordance with the dictates of the climate so as to extend or reduce the growing season. This may involve use of anti-sense or sense regulation.

The nucleic acid according to the invention, such as a *FCA* gene or homologue, may be placed under the control of an externally inducible gene promoter thus placing the timing of flowering under the control of the user. This is advantageous in that flower production, and subsequent

events such as seed set, may be timed to meet market demands, for example, in cut flowers or decorative flowering pot plants. Delaying flowering in pot plants is advantageous to lengthen the period available for transport of the product from the producer to the point of sale and lengthening of the flowering period is an obvious advantage to the purchaser.

In a further aspect the present invention provides a gene construct comprising an inducible promoter operatively linked to a nucleotide sequence provided by the present invention, such as the *FCA* gene or *Arabidopsis thaliana*, a homologue from another plant species, e.g. a *Brassica* such as *Brassica napus*, or any mutant, variant or allele thereof. As discussed, this enables control of expression of the gene. The invention also provides plants transformed with said gene construct and methods comprising introduction of such a construct into a plant cell and/or induction of expression of a construct within a plant cell, by application of a suitable stimulus, an effective exogenous inducer.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible

promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic characteristic. Thus an inducible (or "switchable") promoter may be used which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or switched on) to a level which brings about the desired phenotype.

Suitable promoters include the Cauliflower Mosaic Virus 35S (CaMV 35S) gene promoter that is expressed at a high level in virtually all plant tissues (Benfey et al, 1990a and 1990b); the cauliflower meri 5 promoter that is expressed in the vegetative apical meristem as well as several well localised positions in the plant body, eg inner phloem, flower primordia, branching points in root and shoot (Medford, 1992; Medford et al, 1991) and the *Arabidopsis thaliana* LEAFY promoter that is expressed very early in flower development (Weigel et al, 1992).

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct

which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Plants transformed with the DNA segment containing the sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611). Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may

be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

In the present invention, over-expression may be achieved by introduction of the nucleotide sequence in a sense orientation. Thus, the present invention provides a method of influencing a flowering characteristic of a plant, the method comprising causing or allowing expression of the polypeptide encoded by the nucleotide sequence of nucleic acid according to the invention from that nucleic acid within cells of the plant.

Under-expression of the gene product polypeptide may be achieved using anti-sense technology or "sense regulation".

The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established. Double-stranded DNA is placed under the control of a promoter in a "reverse orientation" such

that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works. See, for example, Rothstein et al, 1987; Smith et al, 1988; Zhang et al, 1992, English et al 1996. The complete sequence corresponding to the coding sequence in reverse orientation need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A suitable fragment may have about 14-23 nucleotides, e.g. about 15, 16 or 17.

Anti-sense regulation may itself be regulated by employing an inducible promoter in an appropriate construct.

Thus, the present invention also provides a method of influencing a flowering characteristic of a plant, the method comprising causing or allowing anti-sense

transcription from nucleic acid according to the invention within cells of the plant.

When additional copies of the target gene are inserted in sense, that is the same, orientation as the target gene, a range of phenotypes is produced which includes individuals where over-expression occurs and some where under-expression of protein from the target gene occurs. When the inserted gene is only part of the endogenous gene the number of under-expressing individuals in the transgenic population increases. The mechanism by which sense regulation occurs, particularly down-regulation, is not well-understood. However, this technique is also well-reported in scientific and patent literature and is used routinely for gene control. See, for example, van der Krol, 1990; Napoli et al, 1990; Zhang et al, 1992.

Thus, the present invention also provides a method of influencing a flowering characteristic of a plant, the method comprising causing or allowing expression from nucleic acid according to the invention within cells of the plant to suppress activity of a polypeptide with ability to influence a flowering characteristic. Here the activity of the polypeptide is preferably suppressed as a result of under-expression within the plant cells.

Modified version of FCA may be used in influencing a flowering characteristic of a plant. For example a mutant identified herein as *fca-1*, *fca-3* or *fca-4* may be

employed. The sequence changes resulting in these mutants and the resulting phenotypes are discussed above.

Promotion of FCA activity to cause early flowering

Mutations that reduce *FCA* activity cause late flowering under both long and short day conditions, indicating *FCA* involvement in promoting flowering constitutively. Double mutant experiments have also indicated that *FCA* function may be required both upstream and downstream of the gene products involved in conferring inflorescence/floral meristem identity eg. *LEAFY*, *APETALA1* and *TERMINAL FLOWER*. Thus *FCA* function may be involved in the ability of meristems to respond to *LEAFY*, *APETALA1* and *TERMINAL FLOWER* gene products.

The fully spliced *FCA* transcript is present at very low abundance in all conditions so far analysed. Although the *fca* mutation is recessive transgenic *fca* plants homozygous for an introduced wild-type *FCA* gene flowered slightly earlier than plants carry one copy (Example 2), suggesting that under some conditions the level of the *FCA* transcript is limiting to flowering time. This indicates that flowering may be manipulated by using foreign promoters to alter the expression of the gene. In addition, the majority of the transcript is present in a form that cannot make active protein. Thus alternative splicing may be a specific control mechanism to maintain relatively low levels of the FCA protein. Alteration of this splicing pattern, for example by introducing an *FCA*

gene lacking introns into plants, may give much higher levels of the FCA protein which in turn would give accelerated flowering.

Causing early flowering under non-inductive or inductive conditions

Wild-type *Arabidopsis* plants flower extremely quickly under inductive conditions and the *FCA* gene is expressed prior to flowering, although at a low level. The level of the *FCA* product may be increased by introduction of promoter, eg CaMV35S or meri 5, fusions. In addition, introduction of an *FCA* gene lacking introns may increase the level of FCA protein and cause early flowering in all conditions.

Inhibition of FCA activity to cause late flowering

fca mutations cause late flowering of Arabidopsis. Transgenic approaches may be used to reduce *FCA* activity and thereby delay or prevent flowering in a range of plant species. A variety of strategies may be employed. This late flowering can then be overcome, if so desired, by giving the imbibed seed or plants of different ages, a vernalization treatment.

Expression of sense or anti-sense RNAs

In several cases the activity of endogenous plant genes has been reduced by the expression of homologous antisense RNA from a transgene, as discussed above.

Similarly, the expression of sense transcripts from a transgene may reduce the activity of the corresponding endogenous copy of the gene, as discussed above. Expression of an antisense transcript from the *FCA* gene has been shown reduce activity of the endogenous gene and cause late flowering (Example 3).

Expression of modified versions of the FCA protein

RNA binding proteins have a modular structure in which amino acid sequences required for binding different RNA molecules are separate domains of the protein (Burd and Dreyfuss 1994). This permits the construction of truncated or fusion proteins that display only one of the functions of the RNA binding protein. In the case of *FCA*, modification of the gene in vitro and expression of modified versions of the protein may lead to dominant inhibition of the endogenous, intact protein and thereby delay flowering. This may be accomplished in various ways, including the following:

Expression of a truncated FCA protein.

Some multi-RNP motif proteins can bind different RNA sequences simultaneously. U1 A for example, binds to U1 small nuclear RNA through its first RNA-binding domain and to pre-mRNA sequences through its second, thus controlling splicing (Burd and Dreyfuss 1994). Expression of an FCA protein with only one of these RNP motifs may dominantly block *FCA* action, by preventing binding of the

full size FCA protein. Also expression of a mutant FCA protein not encoding the C terminal sequences may prevent the correct alignment of the binding of the RNA molecule and so again block wild-type FCA binding.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

In the Figures:

Figure 1 shows a nucleotide sequence according to one embodiment of the invention, being the sequence of the genomic region encoding *FCA* obtained from *Arabidopsis thaliana*. Introns are shown in small letters, exons in capitals. Features: ▼(1118)- transcription start; □(1532-1534, 1568-70, 1601-1603) - putative translation start ATG; ⌐β(2753) - Poly A site of β-transcript; ⊐⊏(7056-7377) - alternative splicing around intron II; __(8771-73) - translation stop TAA; ⌐(9256) - Poly A site. Additional translational stop codon at 3026-3028 within intron 3.

Figure 2 shows the predicted amino acid sequence derived from the nucleotide sequence encoding the *FCA* ORF.

Figure 3 shows the nucleotide sequence of the *FCA* α_B gene, including 5' and 3' flanking sequences. The sequence within the ORF is that of one of the abundant transcripts, that is 18 introns have been spliced out but intron 3 remains. The position of termination of the other abundant transcript is indicated. Primer sequences are given in Table 2. Restriction sites: SalI - 352; HindIII - 776; XbaI - 1157; HindIII - 3125; BglII - 3177; ClaI - 3293; BamHI - 3549; HindIII - 4728; SpeI - 5003. Other important landmarks: 1293-poly A tail added after this nucleotide in cDNA clone 77B or FCA transcript α; 897-5' splice site of intron 3: 2973 3' splice site of intron 3.

Figure 4 compares the *FCA* RRM motifs with those from the Drosophila *SEX-LETHAL* and *TRA-2* genes. Also shown are the C-terminal amino acids with homology to yeast and *C. elegans* proteins.

Figure 5 shows the recombination analysis to position the *FCA* gene.

Figure 6 shows the complementation analysis to localize the *FCA* gene.

Figure 7 shows the complexity and position of the *FCA* gene on the complementing cosmids.

Figure 8 shows the nucleotide sequence of the *Brassica* napus *FCA* homologue and encoded polypeptide: Figure 8a - *Brassica FCA* nucleotide sequence including coding sequence; Figure 8b - polypeptide amino acid sequence encoded by coding sequence of Figure 8a.

Figure 9 shows an alignment of the *Arabidopsis* and *Brassica* FCA amino acid sequences. Topline is *Arabidopsis;* bottom line is *Brassica*.

Figure 10 shows the different transcripts produced from the *FCA* gene. __ open reading frame; * conserved region in *C. elegans* and yeast ESTs; R1, R2 RNA-binding domains 1 and 2.

EXAMPLE 1 - CLONING AND ANALYSIS OF THE FCA GENE

Identification of a 300kb genomic region carrying the FCA gene of Aribidopsis thaliana.

The *fca* mutation had been mapped relative to visible markers to 29cM on chromosome 4. In order to map the locus relative to molecular markers as a starting point for cloning by chromosome walking, the segregation pattern of RFLP markers mapping to the top half of chromosome 4 was analysed in 171 late (homozygous recessive class) flowering individuals from the F2 of a cross between the late flowering mutant *fca*-1 (in a Landsberg erecta background) and the polymorphic early flowering ecotype Columbia. This analysis positioned the *FCA* locus in a 5.2cM interval between markers m326 and m226.

These markers were then used as the starting points for the chromosome walk. YAC clones containing these RFLP markers were identified by colony hybridization experiments. In the initial experiments , the YAC libraries used were the EG, EW and ABI libraries but as

another became available (yUP-May1992) they were incorporated into the analysis. Positively hybridizing YAC clones were confirmed using Southern blot analysis. They were sized using PFGE and Southern blot analysis and then end-probes were generated using either inverse PCR or left-end rescue for use in chromosome walking experiments. In the majority of cases, each step in the walk was covered by two independent YAC clones to avoid false linkages generated by chimaeric YAC clones. These constituted a significant fraction of the EG, EW and yUP libraries and complicated the assembly of the YAC contig. The result of the generation and analysis of 65 end-probes was a YAC contig covering the m326-m226 interval that included 57 YAC clones.

Polymorphisms between Landsberg erecta and Columbia were determined for the left end-probe of EG9D2, right end-probe of YAC clone yUP13C7, right end-probe of YAC clone yUP3F7 and right end-probe of YAC clone EW20B3. Analysis of the segregation pattern of these markers on pooled progeny of recombinants with cross-over points mapping in the m326-m226 interval defined the region carrying the *FCA* gene to between the polymorphisms identified by yUP3F7RE and m226. This interval was covered by two overlapping YAC clones EW20B3 and ABI10C10.

In order to further define the position of the *FCA* gene, more probes were required that mapped within the two overlapping YAC clones. This was achieved by using

end-probes from YAC clones ABI3C4, ABI6C3, a random Sau3A fragment from YAC clone EW20B3 (W5) and two cosmids cAtA2 and g19247. Restriction maps for SmaI, MluI and PacI were constructed and used to position the probes within the YAC clones.

Additional recombinants, where the cross-over point mapped close to the *FCA* locus, were generated by selecting individual plants that were arabinose resistant and had an early/intermediate flowering from the F2 generation of a cross between *fca* (in Landsberg erecta) and ara1 (in Columbia). Progeny of these were checked to confirm that they were homozygous for the arabinose resistance allele and heterozygous for the *fca* mutation. Three of these individuals (A2/7, A1/8 and A4/7) were analysed with the RFLP markers 3F7RE, W5, cAtA2, 19247, 3C4LE, 6C3LE and 226. This defined the north end of the genomic region carrying the *FCA* gene as within the cosmids cAtA2 and 19247. This information is summarized in Fig.5.

Complementation analysis to define the FCA gene.

The two YAC clones EW20B3 and ABI10C10 were gel-purified and hybridized to filters carrying 25500 cosmid clones that contained 15-20kb of *Arabidopsis thaliana* Landsberg erecta genomic DNA. This cosmid library was constructed in a new vector (04541) by cloning a 1.6kb BglII fragment from pHC79 carrying the lambda cos fragment into in the vector pSLJ1711. The resulting

highly stable cosmid cloning vehicle carries Agrobacterium border sequences for transfer of DNA into plant chromosomes, a 35S-NPTII plant selectable marker, lacz-laci sequences for the blue/white insert selection in E.coli and a polylinker with 7 cloning sites.

Positively hybridizing colonies were analysed by hybridizing each clone to Southern blots carrying all the cosmid clones digested with a HindIII, EcoRI and BamHI. This generated a restriction map for the insert of each cosmid and indicated which clones carried overlapping inserts. The cosmids were also run alongside plant DNA and hybridized with the cosmid to confirm that the cosmid insert was colinear with the plant DNA. The two cosmid clones, cAtA2 and cAtB1, mapping to this interval were isolated from a different cosmid library (Olszewski and Ausubel 1988). The result of this analysis was a cosmid contig covering the 300kb interval in which the *FCA* locus had been defined.

Six mutant *fca* alleles were available, two of which had been generated by FN irradiation and one by X-ray irradiation. Irradiation-induced mutations are frequently associated with genomic rearrangements or deletions. In case this would further refine the location of the *FCA* gene, the genomic region covered by the YAC clones EW20B3 and ABI10C10 was examined in all six alleles. The two YAC clones were hybridized to PFGE Southern blots carrying DNA from the different alleles digested with SmaI and MluI. A ~50kb MluI fragment was found to be slightly

smaller in the *fca*-4 allele. Further analysis by hybridization of cosmid clones, corresponding to the region showing the difference, indicated that part of the alteration had occurred in a 1.9kb BamHI fragment carried in cosmids cAtA2 and 19247. This focused our efforts in the first complementation experiments to cosmid clones at the north end of the contig.

Eleven cosmid clones shown in Fig 6, starting with those at the left end, were introduced into the *Arabidopsis fca*-1 mutant using the root explant transformation procedure (Valvekens et al 1988). Seed were collected from self-fertilized kanamycin resistant individuals and analysed with respect to their kanamycin segregation and flowering time. The number of transformants showing complementation to early flowering for each cosmid is shown in Figure 6. The four cosmids that resulted in complementation mapped to the end of the genomic region where the inversion in the *fca*-4 allele mapped.

Identification of the FCA gene.

The complete genomic sequence of Columbia allele corresponding to the genomic region within the complementing cosmid clones was obtained through the efforts of the *Arabidopsis* sequencing initiative centred within this department (G. Murphy pers. comm.). The majority of the genomic region contained in the complementing cosmids is carried on three BamHI

restriction fragments, 4, 1.9 and 2kb. These were isolated and hybridized either separately or pooled to $1x10^6$ phage clones of the PRL-2 cDNA library. This library had been made from pooled RNA samples and was made available by Tom Newman (Michigan). Four clones hybridizing to the 2kb BamHI fragment and 3 to the 4 and 1.9kb fragments were isolated and characterized. They identified two cDNA clones with insert sizes ~1700bp and 1350bp. Analysis of the sizes of the transcripts hybridizing to these two cDNA clones showed that one (in *fca*-4) was reduced in size relative to the other alleles and wild-type and so this cDNA clone was assigned to the *FCA* gene. The other clone showed no differences and was termed 77B

The transcript size of the putative *FCA* gene was >3kb indicating that the cDNA clone was not full length. The cDNA clone was sequenced and found to encode an insert of 1811bp. Primers were designed from the genomic sequence (marked BamX primer on Figure 3) and the 5' end of the cDNA sequence (marked IanRT1 and IanRT2 on Figure 3). First strand cDNA was made using the IanRT2 primer to prime RNA isolated from wild-type seedlings (2 leaf stage). This was used with primers BamX and IanRT2 to PCR amplify a fragment detected as a faint band on an ethidium bromide stained gel. The PCR product was diluted 1/300 and reamplified using primers BamX and IanRT1. The product from this reaction was end-filled using T4 DNA polymerase and cloned into the EcoRV site of the general

cloning vector Bluescript KSII (Stratagene). The product was sequenced and found to be colinear with the genomic sequence and extend the sequence of the cDNA clone by 735bp.

The sequence was compared to all available sequences using BlastX, BlastN and TBlastN. Significant homologies were detected in the TBlastN search to a class of proteins previously defined as RNA binding proteins. The characteristic of these proteins is the presence of one or more RRM motifs made up of conserved amino acids covering an 80 amino acid region (shown in Fig. 4). The positioning of sub-motifs RNP2 and RNP1 and individual conserved amino acids is always maintained within the whole RRM motif. Translation of the sequence of the *FCA* cDNA clone extended in the RT-PCR experiments showed the presence of multiple translation stop codons in the 5' region of the sequence. The first methionine residue downstream of the last translation stop codon and in frame with the rest of the FCA protein was located in the middle of the RRM motif, splitting RNP2 and RNP1. The strong homology of the RRM motif to other RNA binding proteins suggested that this MET residue was not the beginning of the FCA protein. In addition, the transcripts of a large number of RNA-binding proteins are alternatively spliced to yield active and inactive products. The splicing is then regulated, often in an autoregulatory fashion, to control the production of the active protein. These facts suggested that the *FCA*

transcript generated in the RT-PCR experiments contained an intron, just upstream of the RRM motif.

In order to test this hypothesis, several primers were designed from the genomic sequence for use in further RT-PCR experiments. First strand cDNA was made from RNA isolated from seedlings (4 leaf stage), primed with random hexamers (Boehringer). Primers lying within the sequence 5' to the *FCA* cDNA up to the 3' end of the 77B cDNA (the other cDNA clone hybridizing to the complementing cosmid clones), together with IanRT1 gave amplification products of the expected size from the genomic sequence but did not yield smaller products as would be expected from a transcript in which an intervening intron had been spliced out. A primer lying within the 77B cDNA clone marked as cDNAII-BamHI (in Fig.3) was then used in conjunction with the IanRT1 primer. No band was visible on an ethidium bromide stained agarose gel after 30 cycles of amplification. The PCR reaction was then diluted 1/300 and re-amplified using primers cDNAII-1 and RevEx4 (shown in Figure 3). The PCR product was digested with SalI and BglII restriction enzymes and cloned into SalI and BamHI digested BluescriptKSII plasmid. Sequence analysis of the 760bp product and comparison to the genomic sequence revealed that a 2kb intron had been spliced out to join the ORF within the 77B cDNA to that carrying the RRM motif in the *FCA* gene. This splicing revealed the

presence of a second intact RRM motif interrupted by intron 3.

Direct comparison of the *FCA* sequence with that of *LUMINIDEPENDENS* and *CO*, the other flowering time genes cloned from *Arabidopsis* (Lee et al, 1994, Putterill et al 1995), detected no significant homology.

Mutations in the fca mutant alleles.

cDNA was made from RNA isolated from the mutant alleles. This was amplified using cDNAII-BamHI and cDNA-3'a: BamX and IanRT1; fca5'-1 and fca3'-a (positions indicated on Fig 3). The resulting PCR fragments were cloned and sequenced and compared to the sequence of the wild-type Landsberg erecta transcript. The *fca*-1 mutation converted a C nucleotide at position 6861 into a T. Thus a glutamine codon (CAA) is changed into a stop codon (TAA). The *fca*-3 mutation converted a G nucleotide at position 5271 into an A. The effect of this mutation is to alter the 3' splice junction of intron 7 such that a new 3' splice junction is used 28 nucleotides into exon 8. The *fca*-4 mutation is the result of a rearrangement with the break-point at position 4570 (within intron 4).

EXAMPLE 2 - ISOLATION AND SEQUENCE ANALYSIS OF THE BRASSICA NAPUS HOMOLOGUE.

A *Brassica* napus genomic library constructed from Sau3A partially digested DNA cloned into lambda DASH^{R}II/BamHI vector (Stratagene) was obtained. The

library was screened using the 1811bp *FCA* cDNA clone. A clone carrying a 12kb insert was isolated which hybridized to the *FCA* cDNA clone and the 77B cDNA clone. The lambda clone was digested with SalI which released the full length 12kb *Brassica* insert and this was cloned into Bluescript KSII. Restriction fragments of this clone (a combination of EcoRI, SacI and BamHI) were subcloned into BluescriptKSII and sequenced.

The 12kb *Brassica* fragment was also subcloned into the XhoI restriction site of the Agrobacterium binary vector pSLJ1714 (Jones et al 1992), for transformation into the *fca* mutant. When introduced into the *fca*-4 mutation, using root explant transformation, progeny of the transformant segregated early flowering plants. These flowered with a mean of 8.3 leaves compared to wild-type Landsberg erecta grown alongside with 9.1 leaves and *fca*-4 with 24.1 leaves. Thus the *Brassica FCA* gene fully complements the *fca*-4 mutation.

Expression of FCA mRNA

PolyA mRNA was isolated from a range of developmental stages: 2 leaf, 4 leaf, 6 leaf and 10 leaf, roots and inflorescences, fractionated on Northern blots and hybridized with the 1811bp *FCA* cDNA clone. The combined *FCA* transcript γ was present at approximately the same amount in all tissues examined except for the inflorescences where expression was slightly lower. The prematurely polyadenylated transcript β was detected

using 77B cDNA clone as a probe. The β transcript was ~20-fold more abundant than γ_{A+B}. Transcripts α_{A+B} containing intron 3 were not detected on a northern blot and could only be found using RT-PCR.

FCA expression has also been analysed using RNase protection assays. Using a probe (725 bp to 1047 bp from γ_B construct) the γ_{A+B} transcripts were detected at similar levels in a range of developmental stages in both long and short day photoperiods, and at lower levels in rosettes and inflorences of mature plants. The β transcript was at a higher level in these tissues consistent with the northern blot analysis.

METHODS FOR EXAMPLES 1 AND 2

Growth conditions and measurement of flowering time

Flowering time was measured under defined conditions by growing plants in Sanyo Gallenkamp Controlled Environment rooms at 20°C. Short days comprised a photoperiod of 10 hours lit with 400 Watt metal halide power star lamps supplemented with 100 watt tungsten halide lamps. This provided a level of photosynthetically active radiation (PAR) of 113.7 μmoles photons m-2s-1 and a red:far red light ratio of 2.41. A similar cabinet and lamps were used for the long day. The photoperiod was for 10 hours under the same conditions used for short days and extended for a further 8 hours using only the tungsten halide lamps. In this cabinet the combination of lamps used for the 10 hour period provided a PAR of 92.9

μmoles photons m-2 s-1 and a red:far red ratio of 1.49. The 8 hour extension produced PAR of 14.27 μmoles m-2 s-1 and a red:far-red ratio of 0.66.

The flowering times of large populations of plants were measured in both greenhouse and cabinet conditions. Flowering time was measured by counting the number of leaves, excluding the cotyledons, in the rosette and on the inflorescence. Leaf numbers are shown with the standard error at 95% confidence limits. The number of days from sowing to the appearance of the flower bud was also recorded, but is not shown. The close correlation between leaf number and flowering time was previously demonstrated for Landsberg erecta and *fca* alleles (Koorneef et al, 1991).

Cosmid and RFLP markers.

DNA of lambda clones m210, m326, m580, m226 were obtained from Elliot Meyerowitz (Caltech, Pasadena). Total DNA was used as radiolabelled probe to YAC library colony filters and plant genomic DNA blots. Cosmids g10086, g4546, g4108, g19247 were obtained from Brian Hauge and Howard Goodman (MGH, Boston), cultured in the presence of 30 mg/l kanamycin, and maintained as glycerol stocks at - 70°C. Total cosmid DNA was used as radiolabelled probe to YAC library colony filters and plant genomic DNA blots. Cosmid clones cAtA2 and cATB1 were obtained from Chris Cobbett (University of Melbourne) and cultured in the presence of 10mg/l

tetracycline. Cosmid pCITd23 was provided by Elliot Meyerowitz (Caltech, Pasadena), cultured in the presence of 100 μg/ml streptomycin/spectinomycin and maintained as a glycerol stock at - 70° C. pCIT30 vector sequences share homology to pYAC4 derived vectors, and therefore YAC library colony filters were hybridised with insert DNA extracted from the cosmid. Total DNA of pCITd23 was used as radiolabelled probe to plant genomic DNA blots.

YAC libraries.

The EG and ABI libraries were obtained from Chris Somerville (Michigan State University). The EW library was obtained from Jeff Dangl (Max Delbruck Laboratory, Cologne) and the yUP library from Joe Ecker (University of Pennsylvania). Master copies of the libraries were stored at -70°C (as described by Schmidt et al. Aust. J. Plant Physiol. 19: 341-351 (1992)). The working stocks were maintained on selective Kiwibrew agar at 4°C. Kiwibrew is a selective, complete minimal medium minus uracil, and containing 11% Casamino acids. Working stocks of the libraries were replated using a 96-prong replicator every 3 months.

Yeast colony filters.

Hybond-N (Amersham) filters (8cm x 11cm) containing arrays of yeast colony DNA from 8-24 library plates were produced and processed (as described by Coulson et al. Nature 335:184-186 (1988) and modified (as described by

Schmidt and Dean Genome Analysis, vol.4: 71-98 (1992)). Hybridisation and washing conditions were according to the manufacturer's instructions. Radiolabelled probe DNA was prepared by random-hexamer labelling.

Yeast chromosome preparation and fractionation by pulsed field gel electrophoresis (PFGE).

Five millilitres of Kiwibrew was inoculated with a single yeast colony and cultured at 30°C for 24 h. Yeast spheroplasts were generated by incubation with 2.5mg/ml Novozym (Novo Biolabs) for 1 h at room temperature. Then 1 M sorbitol was added to bring the final volume of spheroplasts to 50 µl. Eighty microlitres of molten LMP agarose (1% InCert agarose, FMC) in 1 M sorbitol was added to the spheroplasts, the mixture was vortexed briefly and pipetted into plug moulds. Plugs were placed into 1.5ml Eppendorf tubes and then incubated in 1 ml of 1 mg/ml Proteinase K (Boehringer Mannheim) in 100 mMEDTA, pH 8, 1% Sarkosyl for 4 h at 50°C. The solution was replaced and the plugs incubated overnight. The plugs were washed three times for 30 min each with TE and twice for 30 min with 0.5 x TVBE. PFGE was carried out using the Pulsaphor system (LKB). One-third of a plug was loaded onto a 1% agarose gel and electrophoresed in 0.5 x TBE at 170 V,20 s pulse time, for 36 h at 4°C. DNA markers were concatemers of lambda DNA prepared as described by Bancroft and Wolk, Nucleic A Res. 16:7405-7418 (1988). DNA was visualised by staining with ethidium bromide.

Yeast genomic DNA for restriction enzyme digestion and inverse polymerase chain rection (IPCR).

Yeast genomic DNA was prepared essentially as described by Heard et al. (1989) except that yeast spheroplasts were prepared as above. Finally, the DNA was extracted twice with phenol/chloroform, once with chloroform and ethanol precipitated. The yield from a 5ml culture was about 10μg DNA.

Isolation of YAC left-end probes by plasmid rescue.

Plasmid rescue of YAC left-end fragments from EG, ABI and EW YACs was carried out as described by Schmidt et al. (1992). IPCR was used to generate left and right end fragments using the protocol and primers described in Schmidt et al (1992).

Gel blotting and hybridisation conditions.

Gel transfer to Hybond-N, hybridisation and washing conditions were according to the manufacturer's instructions, except that DNA was fixed to the filters by UV Stratalinker treatment and/or baked at 80°C for 2 h. Radiolabelled DNA was prepared by random hexamer labelling.

RFLP analysis.

Two to three micrograms of plant genomic DNA was prepared from the parental plants used in the crosses and cleaved in a 300 μl volume. The digested DNA was ethanol

precipitated and separated on 0.7% agarose gels and blotted onto Hybond-N filters. Radiolabelled cosmid, lambda or YAC end probe DNA was hybridised to the filters to identify RFLPs.

RNA extractions

RNA was extracted using a method described by Dean et al (1985)

polyA RNA was isolated using the polyAtract^{R} mRNA isolation system (Promega).

DNA extractions

Arabidopsis DNA was performed by a CTAB extraction method described by Dean et al (1992).

Isolation of cDNA by RT-PCR

Total RNA was isolated from whole seedlings at the 2-3 leaf stage growing under long days in the greenhouse. For first strand cDNA synthesis, 10 μg of RNA in a volume of 10 μl was heated to 65°C for 3 minutes, and then quickly cooled on ice. 10 μl of reaction mix was made containing 1 μl of RNAsin, 1 μl of standard dT17-adapter primer (1 μg/μl; Frohman et al, 1988), 4μl of 5x reverse transcriptase buffer (250mM TrisHCl pH8.3, 375mM KCl, 15mM MgCl2), 2μl DTT (100mM), 1μl dNTP (20mM), 1μl reverse transcriptase (200 units, M-MLV Gibco). This reaction mix was then added to the RNA creating a final

volume of 20μl. The mixture was incubated at 42°C for 2 hours and then diluted to 200 μl with water.

10μl of the diluted first strand synthesis reaction was added to 90μl of PCR mix containing 4μl 2.5mM dNTP, 10μl 10xPCR buffer (Boehringer plus Mg), 1μl of a 100ng/μl solution of each of the primers, 73.7μl of water and 0.3μl of 5 units/μl Taq polymerase (Boehringer or Cetus Amplitaq). The reaction was performed at 94°C for 1 minute, 34 cycles of 55°C for 1 minute, 72°C for 2 minutes and then finally at 72°C for 10 minutes.

DNA sequencing

The Sanger method was used to sequence fragments of interest inserted in a Bluescript plasmid vector. Reactions were performed using a Sequenase kit (United States Biochemical Corporation).

Screening the Landsberg erecta cosmid library and the PRL-2 cDNA library.

26000 clones arrayed in microtitre plates were screened by gridding offsets from 16 microtitre plates onto LB-tet (10μg/ml) plates and then taking colony lifts onto Hybond N filters. 1x106 plaques of the CD4-71-PRL2 library (supplied by the *Arabidopsis* Biological Resource Center at Ohio State University) were screened by plating 20 plates of 50000 plaques and then taking plaque lifts onto Hybond N filters.

Transformation of Arabidopsis

The cosmids containing DNA from the vicinity of *FCA* were mobilised into Agrobacterium tumefaciens C58C1, and the T-DNA introduced into *Arabidopsis* plants as described by Valvekens et al, 1988. Roots of plants grown in vitro were isolated and grown on callus-inducing medium (Valvekens et al, 1988) for 2 days. The roots were then cut into short segments and co-cultivated with Agrobacterium tumefaciens carrying the plasmid of interest. The root explants were dried on blotting paper and placed onto callus-inducing medium for 2-3 days. The Agrobacterium were washed off, the roots dried and placed onto shoot inducing medium (Valvekens et al, 1988) containing vancomycin to kill the Agrobacterium and kanamycin to select for transformed plant cells. After approximately 6 weeks green calli on the roots start to produce shoots. These are removed and placed in petri dishes or magenta pots containing germination medium (Valvekens et al, 1988). These plants produce seeds in the magenta pots. These are then sown on germination medium containing kanamycin to identify transformed seedlings containing the transgene (Valvekens et al, 1988).

EXAMPLE 3 - PLANTS HOMOZYGOUS FOR THE T-DNA INSERTION CARRYING FCA FLOWER EARLIER THAN HETEROZYGOTES.

Two transformants of each of the four cosmid clones that complemented the *fca* mutant phenotype were selfed

and seed of late and early flowering individuals were collected and plated on kanamycin-containing medium. All the late flowering progeny were kanamycin sensitive whilst progeny from the early flowering individuals were either homozygous or heterozygous for kanamycin resistance. This demonstrates that the kanamycin marker on the T-DNA carrying the region containing the *FCA* gene completely co-segregated with the early flowering phenotype. Thus, complementation to early flowering was due to sequences within the insert of the cosmid. LN was counted for the early flowering individuals either homozygous or heterozygous for the T-DNA insert.

TABLE 1

cosmid	K/K	K/-
CL58I16	10.3 (9)	13 (4)
	9.7 (4)	10.4 (10)
CL44B23	9,5 (2)	11.8 (6)
	12 (2)	11.1 (6)
cAtA1	14.2 (5)	15 (3)
	9.6 (3)	10.8 (5)
cAtA2	9.1 (7)	9.3 (3)

12.5 (3) 14.4 (7)

Analysis of flowering time (as measured by total LN) in transformants showing complementation of the *fca* mutant phenotype. For each cosmid two independent transformants were analysed. The leaf number was counted on F2 individuals (the number of which is shown in the bracket) which were then selfed and progeny sown on kanamycin-containing medium to establish whether the plant was homozygous (K/K) or heterozygous (K/-) for the T-DNA insert.

The results, shown in Table 1 above, indicate that the homozygotes flowered significantly earlier than the heterozygotes in all 8 transformants analysed. Thus increasing the *FCA* gene dosage and therefore most likely the amount of gene product causes earlier flowering.

EXAMPLE 4 - ANTISENSE EXPERIMENTS.

A 1184bp BamHI (bp3547, Fig 3)/HindIII (bp4731 Fig 3) restriction fragment from the *FCA* cDNA clone was subcloned into the BamHI/HindIII restriction sites of pBluescriptKSII. The insert was released with the enzymes BamHI and XhoI and subcloned into an Agrobacterium binary vector pSLJ6562 (J.Jones, Sainsbury Laboratory). The resulting plasmid contains the CaMV 35S promoter transcribing the *FCA* cDNA fragment to produce antisense RNA, terminated with 3' sequences from the nopaline synthase gene. This plasmid also carries LB and RB Agrobacterium sequences for delivery into plant cells and

and a nos5'-kan-ocs3' fusion to allow kanamycin selection for transformants. The construct was introduced into *Arabidopsis thaliana* ecotype Landsberg erecta using the root explant transformation procedure of Valvekens et al (1988).

Selfed seed from five transformants were collected, sown on kanamycin-containing medium and and 10 kanamycin resistant individuals transplanted to soil. Three of the transformants segregated for a single T-DNA insertion, the other had two or more. Flowering time, assayed as rosette leaf number was measured. Progeny from four of the five transformants were late flowering, producing 12 rosette leaves, compared to 4 for the fifth transformant. Grown alongside, in these particular conditions, non-transformed Landsberg *erecta* and *fca*-1 plants flowered with ~4 and 11 rosette leaves respectively. Thus the antisense construct (as a single locus) effectively reproduced the late flowering phenotype of the *fca*-1 mutation.

EXAMPLE 5 - CONSTRUCTION OF PROMOTER FUSIONS TO THE FCA OPEN READING FRAME.

A genomic SalI-XhoI fragment carrying the whole *FCA* gene plus 64 bp upstream of the putative start of translation and 500 bp downstream of the site of polyadenylation was cloned into the XhoI site of the Agrobacterium binary vector pSLJ 6562 (described above). This resulted in a vector carrying a nos-kan fusion for

transformant selection and a fusion where the 35S promoter is driving the *FCA* genomic region (21 exons, 20 introns). Tranformants have been made using this construct.

This construct when introduced into *fca-4* plants corrected the late flowering phenotype causing the plants to flower with 6.4 leaves under a long-day photoperiod. This was similar to wild-type Landsberg erecta which flowered with 6.2 leaves when grown alongside.

EXAMPLE 6 - CONSTRUCTION OF AN FCA GENE LACKING INTRONS - TRANSCRIPTS γ_A AND γ_B.

The γ_A construct was created by cloning together seven fragments:

i. an EcoRI (a site present to the insert junction in the multiple cloning site of the vector) - SalI fragment from the cosmid CL43B23. This fragment contains the 5' promoter and untranslated region of *FCA* and the 5' region of the ORF.

ii. a 425 bp SalI-HindIII restriction fragment from cDNA clone 77B.

iii. the region of the spliced transcript covering the 5' splice site of intron 3 was generated using RT-PCR with primers cDNAII-BamHI and IanRT1. The product was reamplified using cDNAII-1 and RevEx4, digested with SalI and BglII and cloned into pBluescriptKSII digested with SalI and BamHI. A 270 bp HindIII fragment from this

plasmid was then used in the reconstruction of the fully spliced transcript.

iv. a region of the spliced transcript was amplified using RT-PCR and primers BamX and IanRT1. This was digested with HindIII and BglII and the 52 bp fragment used in the reconstruction of the fully spliced transcript.

v. a region of the spliced transcript was amplified using RT-PCR and primers BamX and Rev404 (position indicated on Fig.3). A 256 bp ClaI - BamHI fragment was released and gel-purified for use in the reconstruction of the fully spliced transcript.

vi. a ClaI-SpeI fragment was excised from the *FCA* cDNA clone (the 1811 bp clone isolated from the PRL-2 library)

vii. a SpeI-XhoI fragment, carrying the last ~140bp of 3' untranslated region plus ~500 bp of 3' genomic sequence, was isolated from the *FCA* genomic clone.

The seven fragments used to construct the *FCA* gene lacking introns were assembled in two parts, 5' region and then 3' region, which were then combined.

A. 5' region. Fragment iv was cloned into pBluescriptKSII as a HindIII/ClaI insert. Fragment ii was then cloned into this as an EcoRI/HindIII fragment (the EcoRI site coming from the multi-cloning site in the cDNA cloning vector). Fragment iii was then cloned into the HindIII site between fragments ii and iv, the correct orientation being determined using an asymmetrically

positioned RsaI site. Fragment i was then cloned into the EcoRI/SalI sites.

B. 3' region. Fragment vii was cloned into the SpeI/XhoI sites present in fragment vi (the XhoI site coming from the multiple cloning site in the vector). Fragment v was then cloned into the BamHI site, the correct orientation being determined using an asymmetrically positioned ClaI site.

The 3' region containing fragments v, vi and vii was then cloned into the plasmid containing the 5' fragments as a ClaI/XhoI fragment.

The γ_B construct was generated by replacing the EcoNI fragment (1503 bp to 2521 bp of spliced transcript) with an EcoNI fragment from a clone derived from RT-PCR from *Ler* RNA that contained the alternatively spliced form encoding the full length protein.

The resulting constructs were released from the vector using EcoRI and XhoI and cloned into the EcoRI/XhoI sites of the Agrobacterium binary vector pSLJ1714 (Jones et al 1992). Transformants carrying this construct have been generated.

Construct γ_A when introduced into Landsberg erecta caused it to flower with 5.6 leaves under a long-day photoperiod. This was slightly earlier than wild-type Landsberg erecta which flowered with 6.2 leaves when grown alongside. When grown under short-day photoperiod 1/4 of the progeny from the tranformant flowered early (with an average of 8.7 leaves). This is significantly

earlier than wild-type Landsberg erecta which flowers with 23.5 leaves under these conditions.

EXAMPLE 7 - EXPRESSION IN E.COLI.

The γ_B construct, described in Example 6, was digested with Sal1 and KpnI and cloned into the XhoI-KpnI sites of the *E. coli* expression vector pRSETC (Invitrogen Corp.). The resulting vector has the *FCA* cDNA cloned in frame with a polyhistidine metal binding domain, which enables the recombinant protein to be purified away from native E.coli proteins using a metal affinity resin (ProBond ™ Ni^{2+}, Invitrogen Corp.). The FCA protein did not bind well to the affinity columns and so was separated from the E.coli proteins by excision from an SDS-polyacrylamide gel. Protein was extracted from the gel slice and used to inject rabbits. A booster jab was given and then two bleeds taken. The antibodies produced detect the FCA protein dot blotted onto nylon membrane at >1/10,000 dilution.

EXAMPLE 8 - PRIMERS DESIGNED TO AMPLIFY GENES CONTAINING RRM DOMAINS WITH HIGH HOMOLOGY TO FCA.

Based on the homology between etr-1, an EST derived from a human brain mRNA (dbest H1995); the *Drosophila* sexlethal protein; the human nervous system proteins HuD, HuC, Hel-N1, and Hel-N2; and the *Xenopus* proteins elrA, elrB, elrC, elrD a set of degenerate PCR primers were designed containing two regions of very high homology.

Amino acid		F	V	G	S	L	N	K		
OLIGO 1	5'	TTT	GTG	GGG	AGG	CTG	AAC	AAG	C	3'
		C	A	A	TCA	T A	T	A		
		T	T	T	T					
		C	C	C	C					

Amino acid	R	G	C	F	V	K	Y		
OLIGO 1	3'	TCC	GAC	GCC	GAA	GCA	GTT	TAT	5'
			A	A	A	A	A	C	
			T		T		T		
			C		C		C		

EXAMPLE 9 - CONSTRUCTION OF FCA DERIVATIVES TO GENERATE DOMINANT NEGATIVE MUTATIONS AND TO ANALYSE THE EXPRESSION AND SPLICING PATTERN OF THE FCA GENE.

A construct expressing the second open reading frame of transcript α_B under the control of the *FCA* promoter, was constructed by deleting the first open reading frame (from 450 bp to 1206 bp). This was done using oligo mutagenesis to introduce a SphI site at the two positions, digesting and religating the vector.

To examine *FCA* expression *FCA* promoter-GUS fusion constructs have been made. *FCA* promoter + exons 1-4 of *FCA* fused to the *β-glucuronidase (GUS)* gene have been constructed to monitor the splicing within intron 3. The entire *FCA* spliced cDNA (γ_B) with *GUS* fused in frame at the C-terminus has been made to monitor *FCA* protein localization within the cell.

EXAMPLE 10 - IDENTIFICATION OF FCA HOMOLOGUES WITHIN THE ARABIDOPSIS GENOME.

A four genome equivalent Landsberg *erecta* cosmid library was screened using low stringency conditions (40oC overnight, 1% SDS, 5 x SSC, 0.5% milk powder) with the complete *FCA* genomic clone. The filters were washed 2 x 20 min at 45°C in 2 x SSC, 0.5% SDS. After exposure they were then rewashed 2 x 20 min, 50°C in 2 x SSC, 0.5% SDS. 61 cosmid clones were picked, plus two negative control cosmids. Five of these were additional *FCA* clones, leaving 56 putative *FCA* homologues. Minipreps were prepared from 10 ml o/n cultures of cosmids, digested with EcoRI, run on 0.8% gels with positive and negative controls on each gel and Southern blotted. The blots were hybridised separately to 77B and *FCA* cDNA (originally called 61A) (Fig. 7) using the conditions described above and then washed at 45°C only.

Of the putative homologues:-

(a) - 2 cosmids hybridized only to 77B

(b) - 11 cosmids hybridized only to 61A

(c) - 31 cosmids hybridized to both cDNAs

(d) - 13 cosmids difficult to score or showed no detectable hybridized

(a) 2 cosmids appear not to be related

(b) - 49 C 22 and 67 I 3 share common EcoRI fragments

- 18 G 16 and 7 L 2 " " "

(c) - 39 G 10, 46 H 15, 56 F 2 and 59 A 8 share common EcoRI fragments,

- 39 G 10 and 56 F 2 share additional frag

- 4 H 4 and 45 K 24 share two frags

- at least nine other pairs of cosmids may have at least one EcoRI fragment in common.

Table 2

Primers	Sequence	bp start Figure 3
cDNAII-BamHI	5' CAGGATCCTTCATCATCTTCGATACTCG 3'	25
cDNAII-1	5' GTCCCTCAGATTCACGCTTC 3'	228
cDNAII-3'a	5' CACTTTTCAAACACATC 3'	1167
cDNAII-3'b	5' GTTCTCTGTACATTAACTC 3'	1213
BamX	5' ATTGAGATTCTTACATACTG 3'	2568
RevEx1A	5' TAAGACATGTCTGACAG 3'	2838
RevEx1B	5' GTGATCTGATTGTGCAG 3'	3030
RevEx4	5' TAGACATCTTCCACATG 3'	3145
IanRT1	5' CAATGGCTGATTGCAACCTCTC	3320
IanRT2	5' TCTTTGGCTCAGCAAACCG 3'	3348
Rev404	5' CAATGTGGCAGAAGATG 3'	3673
fca-3'a	5' AGGCCATTGTTTGGCAGCTC	4941
fca-3'b	5' CCCAGCTAAGTTACTACTAG 3'	5003

REFERENCES

Bancroft *et al.* (1988) Nucl. Acids Res. 16:7405-7418

Benfey *et al.* EMBO J 9: 1677-1684 (1990a).

Becker, *et al.* (1994) The Plant Journal 5, 299-307.

Bell *et al.* (1988) Cell 55, 1037-1046.

Benfey *et al.* EMBO J 9: 1685-1696 (1990b).

Bower *et al.* (1992) The Plant Journal 2, 409-416.

Burd *et al.* (1994) Science 265, 615-621.

Cao *et al.* (1992) Plant Cell Reports 11, 586-591.

Chandler *et al.* (1994) J. Exp. Bot. 45, 1279-1288.

Chang *et al.* (1988) Proc Natl Acad Sci USA 85:6856-6860

Christou *et al.* (1991) Bio/Technol. 9, 957-962.

Chung *et al.* (1994) Plant Mol. Biol. 26, 657-665.

Coulson *et al.* (1988) Nature 335:184-186

Dale *et al.* (1994) Murphy D.J. VCH, Weinheim, Germany.

Datta *et al.* (1990) Bio/Technol. 8, 736-740.

Dean *et al.* (1985) EMBO.J. 4, 3055-3061.

Dean *et al.* (1992) Plant Journal 2, 69-82.

English *et al.* (1996) The Plant Cell. 8, 179-188.

Frohman *et al.* (1988) Proc Natl Acad Sci USA 85, 8998-9002.

Gordon-Kamm *et al.* Plant Cell 2, 603-618.

Heard *et al.* (1989) Nucl Acids Res 17:5861

Jones *et al.* (1992) Transgenic Research 1, 285-297.

Koornneef *et al.* (1991) Mol Gen Genet 229, 57-66.

Koornneef *et al.* (1983) Heredity 74, 265-272.

Koornneef *et al.* (1994) The Plant J. 6, 911-919.

Koziel *et al.* (1993) Bio/Technol. 1, 194-200.

Lee *et al.* (1994) The Plant Cell 6, 75-83.

Lee *et al.* (1994) The Plant J. 6, 903-909.

Mandel *et al.* (1995) Nature 377, 522-524.

Medford, J.I. (1992) Plant Cell 4, 1029-1039.

Medford *et al.* (1991) Plant Cell 3, 359-370.

Mitzukama *et al.* (1992) Cell 71, 119-131.

Moloney *et al.* (1989) Plant Cell Reports 8, 238-242.

Napoli *et al.* (1990) The Plant Cell 2, 279-289.

Olszewski *et al.* (1988) Nucleic Acids Res. 16, 10765-10782.

Potrykus (1990) Bio/Technology 8, 535-542.

Putterill *et al.* (1993) Mol Gen. Genet. 239, 145-157.

Putterill *et al.* (1995) Cell 80, 847-857.

Radke *et al.* (1988) Theoretical and Applied Genetics 75, 685-694.

Rhodes *et al.* (1988) Science 240, 204-207.

Robinow *et al.* (1988) Science 242: 1570-1572.

Rothstein *et al.* (1987) Proc. Natl. Acad. Sci. USA 84, 8439-8443.

Sambrook *et al.* (1989). Molecular Cloning: A laboratory Manual. (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory).

Schmidt *et al.* (1992) Physical mapping of the *Arabidopsis thaliana* genome. Genome Analysis Vol.4 Strategies for physical mapping Cold Spring Harbour Laboratory Press 71-98

Schmidt *et al.* (1992) Aust J Plant Physiol 19: 341-351

Shimamoto *et al.* (1989) Nature 338, 2734-2736.

Smith *et al.* (1988) Nature 334, 724-726.

Somers *et al.* (1992) Bio/Technol. 10, 1589-1594.

Stiekema *et al.* (1988) Plant Molecular Biology 11, 255-269.

van der Krol *et al.* (1990) The Plant Cell 2, 291-299.

Vasil *et al.* (1992) Bio/Technol. 10, 667-674.

Valvekens *et al.* (1988). Proc. Natl. Acad. Sci. USA 87, 5536-5540.

Weigel *et al.* (1992) Cell 69, 843-859.

Weigel *et al.* (1995) Nature 377, 495-500.

Wester *et al.* (1994) Plant J. 5, 261-272.

Worley *et al.* (1995) Genome Research 5: 173-184

Zhang *et al.* (1992) The Plant Cell 4, 1575-1588.

CLAIMS:

1. A nucleic acid isolate comprising a nucleotide sequence coding for a polypeptide comprising the amino acid sequence shown in Figure 2.

2. Nucleic acid according to claim 1 wherein the coding sequence comprises a sequence shown as an exon in Figure 1.

3. Nucleic acid according to claim 2 wherein the coding sequence comprises the sequences shown as exons in Figure 1.

4. Nucleic acid according to claim 1 wherein the coding sequence is a mutant, allele or variant of the coding sequence of Figure 1.

5. Nucleic acid according to any of claims 1 to 3 comprising an intron.

6. Nucleic acid according to claim 5 comprising an intron as shown in Figure 1.

7. Nucleic acid according to claim 6 wherein said intron is intron 3 of Figure 1.

8. A nucleic acid isolate comprising a nucleotide sequence coding for a polypeptide comprising an amino

acid sequence mutant, allele or variant of the FCA amino acid sequence of the species *Arabidopsis thaliana* shown in Figure 2, by way of insertion, deletion, addition or substitution of one or more amino acids, or a homologue from another species, wherein expression of said nucleic acid in a transgenic plant influences a flowering characteristic of said plant.

9. Nucleic acid according to claim 8 wherein said flowering characteristic is the timing of flowering.

10. Nucleic acid according to claim 9 wherein said mutant, allele or variant has the ability to advance flowering in a plant.

11. Nucleic acid according to claim 9 wherein said mutant, allele or variant has the ability to delay flowering in a plant.

12. Nucleic acid according to any of claims 8 to 11 comprising an intron.

13. Nucleic acid according to claim 12 comprising an intron as shown in Figure 1.

14. Nucleic acid according to claim 13 wherein said intron is intron 3 of Figure 1.

15. Nucleic acid according to claim 14 comprising the nucleotide sequence of *FCA* α_B, i.e. that of Figure 3.

16. Nucleic acid according to claim 8 that has the nucleotide sequence of *FCA* α_A, i.e. intron 3 of Figure 1 and all the exons of Figure 1 except for the exon nucleotides indicated in Figure 1 to be within the alternative intron splicing sites around intron 11.

17. Nucleic acid according to claim 8 that has the nucleotide sequence of *FCA* γ_A, i.e. all the exons of Figure 1 except for the exon nucleotides indicated in Figure 1 to be within the alternative intron splicing sites around intron 11.

18. Nucleic acid according to claim 8 wherein said species other than *Arabidopsis thaliana* is a *Brassica*.

19. Nucleic acid according to claim 18 wherein said homologue comprises the amino acid sequence shown in Figure 8b.

20. Nucleic acid according to claim 19 comprising the coding sequence shown in Figure 8a.

21. Nucleic acid according to claim 19 wherein the coding sequence is a mutant, allele or variant of the coding sequence of Figure 8a.

22. A nucleic acid isolate comprising a nucleotide sequence coding for a polypeptide comprising an amino acid sequence mutant, allele or variant of the amino acid sequence encoded by the nucleic acid of claim 19, by way of insertion, deletion, addition or substitution of one or more amino acids, which mutant, allele or variant has at least 80% amino acid identity with the sequence of Figure 8b and ability to influence a flowering characteristic of a plant.

23. Nucleic acid according to any of claims 1 to 22 further comprising a regulatory sequence for expression of said polypeptide.

24. Nucleic acid according to claim 23 comprising an inducible promoter.

25. A nucleic acid isolate comprising a nucleotide sequence complementary to a coding sequence of any of claims 1 to 22, or a fragment of a said coding sequence suitable for use in anti-sense regulation of expression.

26. Nucleic acid according to claim 25 which is DNA and wherein said nucleotide sequence complementary to a said coding sequence or a fragment thereof is under control of a regulatory sequence for anti-sense transcription.

27. Nucleic acid according to claim 26 comprising an inducible promoter.

28. A nucleic acid vector suitable for transformation of a plant cell and comprising nucleic acid according to any preceding claim.

29. A host cell containing heterologous nucleic acid according to any preceding claim.

30. A host cell according to claim 29 which is bacterial.

31. A host cell according to claim 29 which is a plant cell.

32. A plant cell according to claim 31 having said heterologous nucleic acid within its genome.

33. A plant cell according to claim 32 having more than one said nucleotide sequence per haploid genome.

34. A plant comprising a plant cell according to any of claims 31 to 33.

35. Selfed or hybrid progeny or a descendant of a plant according to claim 34, or any part or propagule of such a plant, progeny or descendant, such as seed.

36. A method of influencing a flowering characteristic of a plant, the method comprising causing or allowing expression of the polypeptide encoded by heterologous nucleic acid according to any of claims 1 to 24 within cells of the plant.

37. A method of influencing a flowering characteristic of a plant, the method comprising causing or allowing transcription from heterologous nucleic acid according to any of claims 1 to 24 within cells of the plant.

38. A method of influencing a flowering characteristic of a plant, the method comprising causing or allowing anti-sense transcription from nucleic acid according to any of claims 25 to 27 within cells of the plant.

39. Use of nucleic acid according to any of claims 1 to 24 in the production of a transgenic plant.

40. Use of nucleic acid according to any of claims 25 to 27 in the production of a transgenic plant.

Figure 1

```
   1  gatctaggtg aaattaatct gaagtttaga aatagatttt cttggaactt
  51  cggagaaaat atgcttcact caacttttt ttggtgctat atgaacaaag
 101  ataatggtca tatgaatgta aacgtgtttt gggatgatgt tatcttgttc
 151  catagatgcg gttggaagaa ttgcatttgg actgcaaaaa ctgatggcct
 201  ttatctttgg aattcagcga gcggtgaaga tgtattgtca agaaaatggg
 251  aagttggatg gtgaagccat atttttgctt ttgggtaatt ttttagtaca
 301  tgtatcttgt tgtttttggc aaaaaaaaaa ttgaaataat aaaaaacatt
 351  tgttttaact ttctctctta ttttgtgtat ttttcatcaa tgatagattt
 401  tttgttttag ttctttattt ataggtcatt taattattag attaatttcc
 451  tgagataata agatcataga ttaaataaca atattgtgtt tgtgatatat
 501  agagattaca ttttacactt atatatagtg gtaagatttc tttttgcttt
 551  caaaccatta aaaacctgtt aaacgattaa cttgactcaa gacaaagcca
 601  ttgattattg actcatgaat ctagtgactc atgatgaagg agacgaacag
 651  taaatattca tttgattatt ttaggtaaaa ggtagttcag acctagtcat
 701  atatcctcta aattcatata gtgatgcaag tattttgcat tacttagaac
 751  tttatattat tgatcaccca acacatgatt taataaacgc catgaaatgc
 801  atgtactata tcaaaatgtt tctgaagcat atagttgaca tgagaatttt
 851  ggattggact taagaatgtg agagttacct gaaatgtcaa ttttttccc
 901  tttgttaacg aaaactcatt ggaacaattg tatccccctt ttggcagtat
 951  ataaatatat tgatggccca agtagctgta ttttccgtta tcagccaaga
1001  ctcaataaag tctaccggtc caaatttcaa ctgaatcacc ggtccaacca
1051  ctattaccgt aactagaccg ctttttcttt tttacattcg gacaaaaaaa
1101  tcaaaatttc gagcaactaa attgatctca tcttcaatcA AATTCATCAT
1151  CTTCGATACT CGTTTCTTCT CTCTTTGGTT TCATACAGAT CCCAAATTTC
1201  TAGGGCTCCT AGTCCTTTGA TTTCTTCGAC TGGAATCGCA ATTCCCCACT
1251  ACGTCAAGCT GGACAGACAC CGAAGGGATC GCCATGAGAG TGGCGGCTAC
1301  GAGGATTCCT ACCATAACCA CCGAGCCCAT CCCAGAGGTC CATCTCGTCC
1351  CTCAGATTCA CGCTTCGAAG AGGATGATGA TGATTTTCGC CGCCACCGTC
1401  GTCGTCGTGG AAGCAGCCCT AGCAATTATC GAATTGGAAT TGGGGGCGGA
```

Figure 1 Continued


```
1451  GGAGGAGGTA ATGGTGGTCG ACGCTGGGAA GATGACACTC CTAAGGATTT
1501  TGATGGTCCC GGAGATGGAG GTTTCCGGCA GATGAATGGT CCCCCAGATA
1551  GAGTAGATTT TAAGCCTATG GGTCCTCACC ATGGTGGAAG TTTTCGGCCT
1601  ATGGGGTTTG CCTACGATGA TGGTTTTCGT CCAATGGGTC CTAACGGTGG
1651  TGTGGGAGGA GAAGGGACAC GGTCAATTGT TGGAGCTCGG TATAACTATC
1701  CCGCGAAGTA TCCTCCTTCA GAGAGTCCAG ACAGGAGGAG ATTTATCGGT
1751  AAAGCAATGG AGTCTGATTA TTCTGTAAGA CCGACTACAC CGCCGGTCCA
1801  GCAGCCTCTT TCCGGTCAGA AAAGAGGGTA TCCTATCTCA GACCATGGCA
1851  GCTTTACTGG AACTGgtaag catgagttca ctcttctttc ttctatgtat
1901  atttattctt gtagtctgtt aaggttcctg agtgtctctt atttttgtgg
1951  gaatcaatga ttagagtatt gaaaggtagt atggttgtta tgttactgta
2001  ttgttgaagg tttttcatgg gatcgactct agaggatcct ttcgattttc
2051  ccatgtatgt gataatcaaa actatatgcc atcttcatgt gtatccttat
2101  ctggttaatt tgatttgcag ATGTCTCTGA TCGTAGCAGT ACAGTCAAGC
2151  TTTTTGTTGG ATCTGTACCA AGGACAGCTA CAGAAGAAGA Agtgagttaa
2201  tcttggaaat cattgttatc tatatactca ttactgagaa ccttttctaa
2251  atttttttctg ttggttttca tattgtagAT CCGTCCCTAT TTCGAACAGC
2301  ATGGAAATGT TCTGGAGGTT GCTCTGATCA AGGACAAGAG AACTGGACAG
2351  CAGCAAGgta tgtcaatctc cattttatta ggaaatagtc gtgaattata
2401  ctttttaaaa tttcaggtct ccctgaaaag gctgatggga agcaacccca
2451  gtctcatcat tggcctccaa ttgtttgcaa caattttcgg gcttattgct
2501  tatgcttgcc agcgtcttat ctgtgttcga ttctgtcaca gaagaaggct
2551  acctgtgcta agaaagggtt tatgtactta tgttgggcaa atagatttcg
2601  ctacttgtgt gtattctaga actttagatg tgtttgaaaa gtgtagaatt
2651  tattgagggt gttttagagt tggagttaat gtacagagaa ctgaattttg
2701  ctgttgcctt tatagtggga attggttata agaacatcgc tattttcctc
2751  tcctattgaa attcattttc tttactcttc ctctagatgg attgaagatg
2801  ttgtgtatgg tcttgacagg atgaatgtat ttttttaagt tggtagtttg
2851  ataaggacat gaggttcaaa agatggtttc ttgatttgcc actcctgctg
2901  gtcaaagatt tggccgtctt tctaatttta tcatgttgga ggttttggcgt
```

Figure 1 Continued

2951 ctttcattttc tttcatatca atttatgggt gtgctgtcta ttggttaatg

3001 atggcattcc ttttaccttt ttggatgagt gatgctggaa tgaatgcgtt

3051 tctccttttc ttttgttgat ggcctgagga actatgatgg ctatatttct

3101 ttccactctc tttgaatggc ctgaaatgtg tgcttttctgt atggtcgtcc

3151 ctctcaattt cttggatggc ttgtgatgtg atataccatc tctcgtcata

3201 ggtgaatgaa tgatttgttt agtagttctt atgtatgtat tttgtatgtt

3251 cccacgtctc tattccttgg atggcttgtg atgtgatata ccatctctcg

3301 tcatagatga atgaatattt tgttgagtag ctcttatgtc tgtatggtgg

3351 cccttgcagt gctgatcgat atttatgtgg aagaaatgtt tgatgataga

3401 tttttttttgt atgctccctt ttcgctaatc aagcctttgt gcttgcaagg

3451 tgcaactgtt attttattat tgaatttcct gttctactac tccatttagt

3501 tctgtctcta ttttgtcagt gtgaagaaat actagacgat gaatggtgtg

3551 tttgtacgtg catagttatt tataaattct tgactttcca agaagttatt

3601 atttctataa ctgctacacc tttgtggatg gcagaacaaa tgcatctgat

3651 tgtggtgaca taaacacttt tgatcgcggt tgaatgtact agattccata

3701 caactctttc ttcagccttg tgaaatatta ttatgttagg tggtgcaaac

3751 atatggaagg aacctgattg ttttagtttc ttagaatagt ttctgatgtt

3801 aatacagcat gttgacttca ctctcttgcc cttgatcaat cagcatcagg

3851 caggggccta attatgtatt acatgaagca atcgtattct tttctgaatt

3901 agatttttttt ccaatgagtt atcttgccca taactgtagt tctttatttg

3951 aagtcttcaa atgcttgatg tatggtgacg aaaatgtgta tatgttttgg

4001 ttttgattat ccgctactca tcaattattg agattcttac atactgaatc

4051 cgttactttg gacctatagt tatgttttat gttgctaatt aacttgtaca

4101 tgtttctaga ttttctttca aatggatcct gcttggacaa atgcagccac

4151 cctttgtctg aaaggccctc ttgtagatat gttatctgca gatactgact

4201 gtgttcaatt ttttaatatt tgtttttgcc atattctcca tttgaagaca

4251 ttaatttatt ctctccaaca actttacatc aatatttaag tggaggctgt

4301 cagacatgtc ttatgatttt cctactgaac ttatgtgctt tgagtagtac

4351 atcttgttac tagtacaatt tgatggtaga aggaaaagtt gaaccctgaa

4401 acagatagct taagtatcag tcttttaatgc agGCTGTTGT TTTGTAAAAT

4451 ATGCAACTTC GAAAGATGCG GATAGAGCCA TCAGAGCACT GCACAATCAG

Figure 1 Continued

4501 ATCACTCTTC CTGGGgtaat taccctgagg ctttctctta tcaagaacag
4551 gaaactatag gttgtttcac cttttataat tttgttgatt cccagGGAAC
4601 TGGTCCTGTT CAAGTTCGAT ATGCTGACGG GGAGAGAGAA CGCATAGgta
4651 atcaacttcc acacagagta tctaatgtgg ctgtcattgt ctagtgttca
4701 tagccaagac catacgctgc ataagttcag attacaaaaa ttaagaaaat
4751 gtgggaaatg atatgaactt tatggatgtt gatccttttc tttccctgtt
4801 ttctttgcct tactatcaag tgatatagtt ctcttcttct gaagGCACCC
4851 TAGAGTTTAA GCTTTTTGTT GGTTCACTAA ACAAGCAAGC CACTGAAAAA
4901 GAAGTTGAGG AGgtatgttt cgtatcttac tttttgaagt tgttacttat
4951 gtcagattaa cggaacaggg aagagttcta aacttggata ttattgtgtc
5001 ccctgttacc tgagttgata attttaaatg actctttgat aaattttgtt
5051 agtcttacca aagggtgagt gtctagaaaa tctgtgtcaa taatgcaagc
5101 gcttggacat tctacttact gtgtaatctc ttcttccaat tgatccaact
5151 gtttgactgt cataatagat aaaattaata aatgtgaacg gctaccttcc
5201 cagttcaact tatgtgtttc aatttctcat gtaatctttt aacaaactgt
5251 tttattgtta ttgctttaac agATCTTTTT ACAATTTGGT CATGTGGAAG
5301 ATGTCTATCT CATGCGGGAT GAATATAGAC AGAGTCGTGg tatgttttgt
5351 aatttgtact agattctata aattatttgt tgtgtgatga tgttgagatg
5401 gtgaaactgt gtttttcact ttgtagGATG TGGGTTTGTT AAATATTCAA
5451 GCAAAGAGAC GGCAATGGCA GCTATCGATG GTCTCAACGG AACTTATACC
5501 ATGAGAGtaa gctgtgaatc acataagtat ctcagtttct ctcattatca
5551 ccctttggac ctgttttgtt tactggcctc tatcctttcc ccagGGTTGC
5601 AATCAGCCAT TGATTGTTCG GTTTGCTGAG CCAAAGAGGC CTAAACCTGG
5651 CGAGTCAAGg taatgccttg ggtactatat tttgattaat cctaatactc
5701 ttatcaagta aattgtatat accttcattc tttgttctgt ctgagttata
5751 tttgtggaga atcttttgga catggtggag agttgggaac cctgttcctt
5801 ctccagttat tactggaatg tgaagcattg ctttctagat atccttaagt
5851 agttttctgtt tccagGGAAA TGGCACCTCC TGTTGGACTT GGTTCAGGGC
5901 CTCGTTTTCA AGCTTCAGGA CCAAGgtaac tggtgtgaaa ggagatcatg
5951 attatgctca ttaggtaatt atatatgttg acttaccccg gtctcctcat

Figure 1 Continued

6001 ctctatttgt tagGCCTACC TCTAACTTTG GTGACTCTAG TGGGGATGTA
6051 AGCCACACAA ATCCTTGGCG TCCAGCTACT TCACGAAACG TAGGCCCACC
6101 TAGTAACACT GGGATCCGTG GTGCCGGTAG TGACTTTCCC CCTAAACCAG
6151 gtcaagcaac attgccttca aatcaggtga gaacaggttg atgatcatgt
6201 atatcatctt aaatctgcac attcatataa gtaagcgcat agagtttgca
6251 tgtattgtgc gagacaaata aaaagaaagt acttcatata ctgcacacat
6301 gggcttatga caggtgaaaa gaagcatgaa gttctgacct ttcaactttt
6351 catataatgc aacaaacacg atgtgtgttg ctcaaatgat atggccttaa
6401 tttgcagttt gtcagttact gaggcaattt ttttttgaa taatttctag
6451 ccctgatgtg agcttttta aatgtaacat tctatattgt tagGGTGGCC
6501 CGTTAGGTGG TTATGGTGTT CCTCCCCTTA ACCCTCTCCC AGTCCCTGGA
6551 GTTTCATCTT CTGCCACATT GCAACAGgta ctttagctat atttttccaa
6601 ttaagcaaat ctgaaaatgt tgtgatgatt aacttggatt ttcaattgtt
6651 tctattccat agCAAAATCG GGCAGCTGGC CAGCATATAA CACCATTAAA
6701 AAAACCTCTT CACAGTCCAC AGGGTCTCCC TCTCCCCCTC CGTCCGCAAA
6751 CTAATTTCCC TGGGGCCCAG GCACCCTTGC AGAATCCTTA TGCTTATAGC
6801 AGCCAGTTGC CTACCTCTCA GCTGCCACCA CAGCAAAACA TCAGTCGTGC
6851 AACTGCTCCT CAAACTCCTT TGAACATTAA TCTACGGCCA ACAACTGTGT
6901 CTTCTGCAAC TGTTCAATTT CCCCCTCGTT CCCAGCAGCA ACCGCTACAA
6951 AAGATGCAAC ATCCTCCTTC TGAGCTAGCT CAGCTCTTGT CGCAGCAAAC
7001 TCAGAGTCTA CAAGCAACAT TCCAATCGTC TCAGCAAGCA ATTTCTCAGC
7051 TGCAGCAGCA GGTGCAGTCT ATGCAGCAAC CAAACCAAAA TTTACCACTC
7101 TCACAGAATG GCCGAGCTGG TAAACAACAG gtatgaatat agtctctcag
7151 ttgcatctgc ccagacgggt tcttcagctg ctattgtgtt gttttaactt
7201 aaaattattt cctgatagac atcccgtttt ttatccttca tgtgttttag
7251 tattctcccc ttttctaatg ttcctctcgg ctgcttcttt atcagTGGGC
7301 TGGATCTGCA ATTCCAAGAG TGGCTAGCAC CACTGGTTCG ACACCAGTGA
7351 GCTATGTGCA AACAGCTGCA CCTGCAGTAA GTCAGAGCGT AGGTTCTGTC
7401 AAATGTACCT GGACCGAGCA TACCTCGCCT GATGGATTTA AATATTATTA
7451 CAATGGTCTA ACGGGTGAAA GCAAGgtgag aaacgtggtt cctcttttaat
7501 atatttcctt gtgagtttca ggagtattcc tcctggttta ttgtgctatt

Figure 1 Continued

7551 gataatcctt acacatgtat attttatatt tgaagtcctt cagtacgtgc

7601 catattatgt atataattca cttttgcagT GGGAAAAACC TGAGGAAATG

7651 ATAGTGTTCG AACGAGAGCA ACAGAAACAG CAACAACATC AAGAGAAGCC

7701 AACTATACAG CAGTCCCAGA CCCAATTACA GCCGTTGCAG CAACAACCAC

7751 AACAAGTTCA GCAGCAATAT CAGGGCCAGC AATTACAGCA GCCGTTTTAT

7801 TCTTCACTGg ttggtttcgt tttcatgctg gttacattca aatatttttg

7851 tcacatggtt tctaatttgc atatttactc ttgttcattt ggagttgcag

7901 tatccaactc caggggccag ccataatact caggtgtata tctgtttaat

7951 ctgtttactt atttttcatt tcaagatttg attcttgata tgctaatctt

8001 gtggtagaag gagattgacc accttaaagt aaaattcagt agccatggtt

8051 ttgccagcat tttgaaatac agataacaaa tctctaacgt gaatgcctat

8101 tttccttttct aaaatgcagT ATCCATCATT GCCAGTAGGT CAAAATAGCC

8151 AGgtacatat ctgaatctgt ggacttattt ttcattgaac tgattgattc

8201 tcagttacaa cattgacttc ctctgatgcg tagtttttgt aacatatcag

8251 aataacaaaa acttcatctg attcgtatat tctctggttg aaaatctttt

8301 tttctttttct ggaaaatgca gTTTCCTATG TCAGGAATTG GTCAGAATGC

8351 TCAGgtatat atctcatttt gtattaacaa tttcccatac cttctgtacc

8401 tttgaattta atcacagaac ataatgagtt cttggattta atgtcatttt

8451 aaaaagaaac atcagtgata tgacttcctt ccttggttaa aaatggttta

8501 ggcagagctt attttctatt ctgtttggat tgtctagGAT TATGCTCGGA

8551 CACATATACC CGTGGGAGCT GCTTCAATGA ATGATATATC AAGAACTCCA

8601 CAGgtagtta tggtttttat cagtgattca gaacttctct ctgttcataa

8651 ttcgtccttt ggtattcaga tgttcttttt cgttgaaacc gttttttttcc

8701 ttaattctct ttacaatcat atctcttttt cccagAGCCG TCAATCTCCC

8751 CAAGAACTCA TGTGGAAGAA TAAAGCTTGA Ggttcatatc taccctttct

8801 ctcctctctc ttgtattttc tccataccga aacacattcc aatgtatgtg

8851 gttttctttag ttgaagttac ctctgtgttg atcgatactc tacttcagGT

8901 ACATGAGACG AGGAGCTAAA CTATCTCAGT AGCTAGATAG AAATTTCTGG

8951 AACTAATTAG TCAAGGAGAG GAAAAGCAGC AATGGTAGTG TCCTTAGTCT

9001 CTGATTTTTT TAGTTAACCC CTTCAGTTAT AATAGATAGG CGATCGTAGA

Figure 1 Continued

```
9051  CCATCTGCAT TCTATCTTTT CTCTAATCAG ATATCTCCTC CTTTTCCATT
9101  TTAAGAGCTG CCAAACAATG GCCTGTTGTA ACATAGCTAG CGCAAGTTAT
9151  GTCTCATGTT GTGTTACTAG TAGTAACTTA GCTGGGTAAA CCAAACTTTG
9201  ATCCAGATTA GGAGTCATAT ATAATTATAT AAATAGAATA TGTACATTCA
9251  TAGATAgctc atcacttata atgagactag atcttagcaa aatccaactc
9301  taattgtcat tttcagagat ctatcaattt gtagtttcct gatcttcata
9351  tatgtgttcg ctcttctaat gattacgtaa aatcagagtc ctacgtaggt
9401  ggacttcttt aatttttata tagataatta gatatcattc aataagtcgg
9451  gcttttattt ttagttaatc attctacaat tcttcctaat ctcgctatta
9501  ctaccaccgg gtatccctcc cattttaacc atagcgttct taaaatcctc
9551  aaagaaaacc gactgatctg ttgcgtaggt ctcaacaatc gcccttgtcc
9601  ctgggtcttg aaccgctaaa gcctggtctg atggaagcaa tccctcaccc
9651  gagaggaggt ttacatagta ctggttgtca aatgttgatg gagtcaccaa
9701  gtcaagctga gtgataccta cactgggccc aacagtcgag cataactgtt
9751  gcagtgactc gag
```

Figure 2

```
  1  MNGPPDRVDF KPMGPHHGGS FRPMGFAYDD GFRPMGPNGG VGGEGTRSIV
 51  GARYNYPAKY PPSESPDRRR FIGKAMESDY SVRPTTPPVQ QPLSGQKRGY
101  PISDHGSFTG TDVSDRSSTV KLFVGSVPRT ATEEEIRPYF EQHGNVLEVA
151  LIKDKRTGQQ QGCCFVKYAT SKDADRAIRA LHNQITLPGG TGPVQVRYAD
201  GERERIGTLE FKLFVGSLNK QATEKEVEEI FLQFGHVEDV YLMRDEYRQS
251  RGCGFVKYSS KETAMAAIDG LNGTYTMRGC NQPLIVRFAE PKRPKPGESR
301  DMAPPVGLGS GPRFQASGPR PTSNFGDSSG DVSHTNPWRP ATSRNVGPPS
351  NTGIRGAGSD FSPKPGQATL PSNQGGPLGG YGVPPLNPLP VPGVSSSATL
401  QQENRAAGQH ITPLKKPLHS PQGLPLPLRP ETNFPGGQAP LQNPYAYSSQ
451  LPTSQLPPQQ NISRATAPQT PLNINLRPTT VSSATVQFPR RSQQQPLQKM
501  QHPPSELAQL LSQQTQSLQA TFQSSQQAIS QLQQQVQSMQ QPNQNLPLSQ
551  NGRAGKQQWA GSAIPRVAST TGSTPVSYVQ TAAPAVSQSV GSVKCTWTEH
601  TSPDGFKYYY NGLTGESKWE KPEEMIVFER EQQKQQQHQE KPTIQQSQTQ
651  LQPLQQQPQQ VQQQYQGQQL QQPFYSSLYP TPGASHNTQY PSLPVGQNSQ
701  FPMSGIGQNA QDYARTHIPV GAASMNDISR TQQSRQSPQE LMWKNKT
```

Figure 3

```
   1  AAATTGATCT CATCTTCAAT CAAATTCATC ATCTTCGATA CTCGTTTCTT
  51  CTCTCTTTGG TTTCATACAG ATCCCAAATT TCTAGGGCTC CTAGTCCTTT
 101  GATTTCTTCG ACTGGAATCG CAATTCCCCA CTACGTCAAG CTGGACAGAC
 151  ACCGAAGGGA TCGCCATGAG AGTGGCGGCT ACGAGGATTC CTACCATAAC
 201  CACCGAGCCC ATCCCAGAGG TCCATCTCGT CCCTCAGATT CACGCTTCGA
 251  AGAGGATGAT GATGATTTTC GCCGCCACCG TCGTCGTCGT GGAAGCAGCC
 301  CTAGCAATTA TCGAATTGGA ATTGGGGGCG GAGGAGGAGG TAATGGTGGT
 351  CGACGCTGGG AAGATGACAC TCCTAAGGAT TTTGATGGTC CCGGAGATGG
 401  AGGTTTCCGG CAGATGAATG GTCCCCCAGA TAGAGTAGAT TTTAAGCCTA
 451  TGGGTCCTCA CCATGGTGGA AGTTTTCGGC CTATGGGGTT TGCCTACGAT
 501  GATGGTTTTC GTCCAATGGG TCCTAACGGT GGTGTGGGAG GAGAAGGGAC
 551  ACGGTCAATT GTTGGAGCTC GGTATAACTA TCCCGCGAAG TATCCTCCTT
 601  CAGAGAGTCC AGACAGGAGG AGATTTATCG GTAAAGCAAT GGAGTCTGAT
 651  TATTCTGTAA GACCGACTAC ACCGCCGGTC CAGCAGCCTC TTTCCGGTCA
 701  GAAAAGAGGG TATCCTATCT CAGACCATGG CAGCTTTACT GGAACTGATG
 751  TCTCTGATCG TAGCAGTACA GTCAAGCTTT TTGTTGGATC TGTACCAAGG
 801  ACAGCTACAG AAGAAGAAAT CCGTCCCTAT TTCGAACAGC ATGGAAATGT
 851  TCTGGAGGTT GCTCTGATCA AGGACAAGAG AACTGGACAG CAGCAAGGTA
 901  TGTCAATCTC CATTTTATTA GGAAATAGTC GTGAATTATA CTTTTTAAAA
 951  TTTCAGGTCT CCCTGAAAAG GCTGATGGGA AGCAACCCCA GTCTCATCAT
1001  TGGCCTCCAA TTGTTTGCAA CAATTTTCGG GCTTATTGCT TATGCTTGCC
1051  AGCGTCTTAT CTGTGTTCGA TTCTGTCACA GAAGAAGGCT ACCTGTGCTA
1101  AGAAAGGGTT TATGTACTTA TGTTGGGCAA ATAGATTTCG CTACTTGTGT
1151  GTATTCTAGA ACTTTAGATG TGTTTGAAAA GTGTAGAATT TATTGAGGGT
1201  GTTTTAGAGT TGGAGTTAAT GTACAGAGAA CTGAATTTTG CTGTTGCCTT
1251  TATAGTGGGA ATTGGTTATA AGAACATCGC TATTTTCCTC TCCCTATTGA
1301  AATTCATTTT CTTTACTCTT CCTCTAGATG GATTGAAGAT GTTGTGTATG
```

Figure 3 Continued

```
1351  GTCTTGACAG GATGAATGTA TTTTTTTAAG TTGGTAGTTT GATAAGGACA
1401  TGAGGTTCAA AAGATGGTTT CTTGATTTGC CACTCCTGCT GGTCAAAGAT
1451  TTGGCCGTCT TTCTAATTTT ATCATGTTGG AGGTTTGGCG TCTTCATTTT
1501  CTTTCATATC AATTTATGGG TGTGCTGTCT ATTGGTTAAT GATGGCATTC
1551  CTTTTACCTT TTTGGATGAG TGATGCTGGA ATGAATGCGT TTCTCCTTTT
1601  CTTTTGTTGA TGGCCTGAGG AACTATGATG GCTATATTTC TTTCCACTCT
1651  CTTTGAATGG CCTGAAATGT GTGCTTTCTG TATGGTCGTC CCTCTCAATT
1701  TCTTGGATGG CTTGTGATGT GATATACCAT CTCTCGTCAT AGGTGAATGA
1751  ATGATTTGTT TAGTAGTTCT TATGTATGTA TTTTGTATGT TCCCACGTCT
1801  CTATTCCTTG GATGGCTTGT GATGTGATAT ACCATCTCTC GTCATAGATG
1851  AATGAATATT TTGTTGAGTA GCTCTTATGT CTGTATGGTG GCCCTTGCAG
1901  TGCTGATCGA TATTTATGTG GAAGAAATGT TTGATGATAG ATTTTTTTTG
1951  TATGCTCCCT TTTCGCTAAT CAAGCCTTTG TGCTTGCAAG GTGCAACTGT
2001  TATTTTATTA TTGAATTTCC TGTTCTACTA CTCCATTTAG TTCTGTCTCT
2051  ATTTTGTCAG TGTGAAGAAA TACTAGACGA TGAATGGTGT GTTTGTACGT
2101  GCATAGTTAT TTATAAATTC TTGACTTTCC AAGAAGTTAT TATTTCTATA
2151  ACTGCTACAC CTTTGTGGAT GGCAGAACAA ATGCATCTGA TTGTGGTGAC
2201  ATAAACACTT TTGATCGCGG TTGAATGTAC TAGATTCCAT ACAACTCTTT
2251  CTTCAGCCTT GTGAAATATT ATTATGTTAG GTGGTGCAAA CATATGGAAG
2301  GAACCTGATT GTTTTAGTTT CTTAGAATAG TTTCTGATGT TAATACAGCA
2351  TGTTGACTTC ACTCTCTTGC CCTTGATCAA TCAGCATCAG GCAGGGGCCT
2401  AATTATGTAT TACATGAAGC AATCGTATTC TTTTCTGAAT TAGATTTTTT
2451  TCCAATGAGT TATCTTGCCC ATAACTGTAG TTCTTTATTT GAAGTCTTCA
2501  AATGCTTGAT GTATGGTGAC GAAAATGTGT ATATGTTTTG GTTTTGATTA
2551  TCCGCTACTC ATCAATTATT GAGATTCTTA CATACTGAAT CCGTTACTTT
2601  GGACCTATAG TTATGTTTTA TGTTGCTAAT TAACTTGTAC ATGTTTCTAG
2651  ATTTTCTTTC AAATGGATCC TGCTTGGACA AATGCAGCCA CCCTTTGTCT
2701  GAAAGGCCCT CTTGTAGATA TGTTATCTGC AGATACTGAC TGTGTTCAAT
2751  TTTTTAATAT TTGTTTTTGC CATATTCTCC ATTTGAAGAC ATTAATTTAT
```

Figure 3 Continued

2801 TCTCTCCAAC AACTTTACAT CAATATTTAA GTGGAGGCTG TCAGACATGT
2851 CTTATGATTT TCCTACTGAA CTTATGTGCT TTGAGTAGTA CATCTTGTTA
2901 CTAGTACAAT TTGATGGTAG AAGGAAAAGT TGAACCCTGA AACAGATAGC
2951 TTAAGTATCA GTCTTTAATG CAGGCTGTTG TTTTGTAAAA TATGCAACTT
3001 CGAAAGATGC GGATAGAGCC ATCAGAGCAC TGCACAATCA GATCACTCTT
3051 CCTGGGGGAA CTGGTCCTGT TCAAGTTCGA TATGCTGACG GGGAGAGAGA
3101 ACGCATAGGC ACCCTAGAGT TTAAGCTTTT TGTTGGTTCA CTAAACAAGC
3151 AAGCCACTGA AAAAGAAGTT GAGGAGATCT TTTTACAATT TGGTCATGTG
3201 GAAGATGTCT ATCTCATGCG GGATGAATAT AGACAGAGTC GTGGATGTGG
3251 GTTTGTTAAA TATTCAAGCA AAGAGACGGC AATGGCAGCT ATCGATGGTC
3301 TCAACGGAAC TTATACCATG AGAGGTTGCA ATCAGCCATT GATTGTTCGG
3351 TTTGCTGAGC CAAAGAGGCC TAAACCTGGC GAGTCAAGGG ACATGGCACC
3401 TCCTGTTGGA CTTGGTTCAG GGCCTCGTTT TCAAGCTTCA GGACCAAGGC
3451 CTACCTCTAA CTTTGGTGAC TCTAGTGGGG ATGTAAGCCA CACAAATCCT
3501 TGGCGTCCAG CTACTTCACG AAACGTAGGC CCACCTAGTA ACACTGGGAT
3551 CCGTGGTGCC GGTAGTGACT TTTCCCCTAA ACCAGGTCAA GCAACATTGC
3601 CTTCAAATCA GGGTGGCCCG TTAGGTGGTT ATGGTGTTCC TCCCCTTAAC
3651 CCTCTCCCAG TCCCTGGAGT TTCATCTTCT GCCACATTGC AACAGGAAAA
3701 TCGGGCAGCT GGCCAGCATA TAACACCATT AAAAAAACCT CTTCACAGTC
3751 CACAGGGTCT CCCTCTCCCC CTCCGTCCGG AAACTAATTT CCCTGGGGGC
3801 CAGGCACCCT TGCAGAATCC TTATGCTTAT AGCAGCCAGT TGCCTACCTC
3851 TCAGCTGCCA CCACAGCAAA ACATCAGTCG TGCAACTGCT CCTCAAACTC
3901 CTTTGAACAT TAATCTACGG CCAACAACTG TGTCTTCTGC AACTGTTCAA
3951 TTTCCCCCTC GTTCCCAGCA GCAACCGCTA CAAAAGATGC AACATCCTCC
4001 TTCTGAGCTA GCTCAGCTCT TGTCGCAGCA AACTCAGAGT CTACAAGCAA
4051 CATTCCAATC GTCTCAGCAA GCAATTTCTC AGCTGCAGCA GCAGGTGCAG
4101 TCTATGCAGC AACCAAACCA AAATTTACCA CTCTCACAGA ATGGCCGAGC
4151 TGGTAAACAA CAGTGGGCTG GATCTGCAAT TCCAAGAGTG GCTAGCACCA

Figure 3 Continued

```
4201  CTGGTTCGAC ACCAGTGAGC TATGTGCAAA CAGCTGCACC TGCAGTAAGT
4251  CAGAGCGTAG GTTCTGTCAA ATGTACCTGG ACCGAGCATA CCTCGCCTGA
4301  TGGATTTAAA TATTATTACA ATGGTCTAAC GGGTGAAAGC AAGTGGGAAA
4351  AACCTGAGGA AATGATAGTG TTCGAACGAG AGCAACAGAA ACAGCAACAA
4401  CATCAAGAGA AGCCAACTAT ACAGCAGTCC CAGACCCAAT TACAGCCGTT
4451  GCAGCAACAA CCACAACAAG TTCAGCAGCA ATATCAGGGC CAGCAATTAC
4501  AGCAGCCGTT TTATTCTTCA CTGTATCCAA CTCCAGGGGC CAGCCATAAT
4551  ACTCAGTATC CATCATTGCC AGTTGGTCAA AATAGCCAGT TTCCTATGTC
4601  AGGAATTGGT CAGAATGCTC AGGATTATGC TCGGACACAT ATACCCGTGG
4651  GAGCTGCTTC AATGAATGAT ATATCAAGAA CTCAACAGAG CCGTCAATCT
4701  CCCCAAGAAC TCATGTGGAA GAATAAAACT TGAGGTACAT GAGACGAGGA
4751  GCTAAACTAT CTCAGTAGCT AGATAGAAAT TTCTGGAACT AATTAGTCAA
4801  GGAGAGGAAA AGCAGCAATG GTAGTGTCCT TAGTCTCTGA TTTTTTTAGT
4851  TAACCCCTTC AGTTATAATA GATAGGCGAT CGTAGACCAT CTGCATTCTA
4901  TCTTTTCTCT AATCAGATAT CTCCTCCTTT TTCATTTTAA GAGCTGCCAA
4951  ACAATGGCCT GTTGTAACAT AACTAGCGCA AGTTATGTCT CATGTTGTGT
5001  TACTAGTAGT AACTTAGCTG GGTAAACCAA ACTTTGATCC AGATTAGGAG
5051  TCATATATAA TTATATAAAT AGAATATGTA CATTCATAGA TAAAAAAAAA
5101  AAAAAAAAAA AAA
```

Figure 4

RNA Binding Domain

```
                                    RNP 2                                              RNP 1
Protein     Domain
fca           1     DVSDRSSTVK LFVGSV PRTATEEEIRPYFEQHGNVLEVALIKDKRTGQQ QGCCFVKY ATSKDADRAIRALHNQITLPG
fca           2     RERIGTLEFK LFVGSL NKQATEKEVEEIFLQFGHVEDVYLMRDEYRQS  RGCGFVKY SSKETAMAAID        GLNG
Sex lethal    1     MNDPRASNTN LIVNYL PQDMTDRELYALFRAIGPINTCRIMRDY       KTGYSFGY  AFVDFTSEMDSQRAIKVLNG
              2     PGGESIKDTN LYVTNL PRTITDDQLDTIFGKYGSIVQKNILRDKLTGRP RGVAFVRY NKREEAQEAISALNNVIPEGG
cra-2         -     SREHPQASRC IGVFGL NTNTSQHKVRELFNKYGPIERIQMVIDAQTQRS RGFCFIYF EKLSDARAAKD SCSGIEVDG

                               LFVGNL     E   V    F   FG I          K    KGFGFVXF          A        L G
                               IYIKG      D             Y  V          R    R YA   Y                   I
```

Homology in C-terminal region

```
fca                 WTEHTSPDGFKYYYNGLTGESKWEKPEEMIVFEREQ
Yeast               WKEAKDASGRIYYYNTLTKKSTWEKPKELISQEELL
C.elegans           WKEFMSDDGKPYYYNTLTKKTQWVKPDGEEITKGEQ

                    W E      G  YYYN LT     W KP
```

Fig.5.

Recombination events defining the position of fca

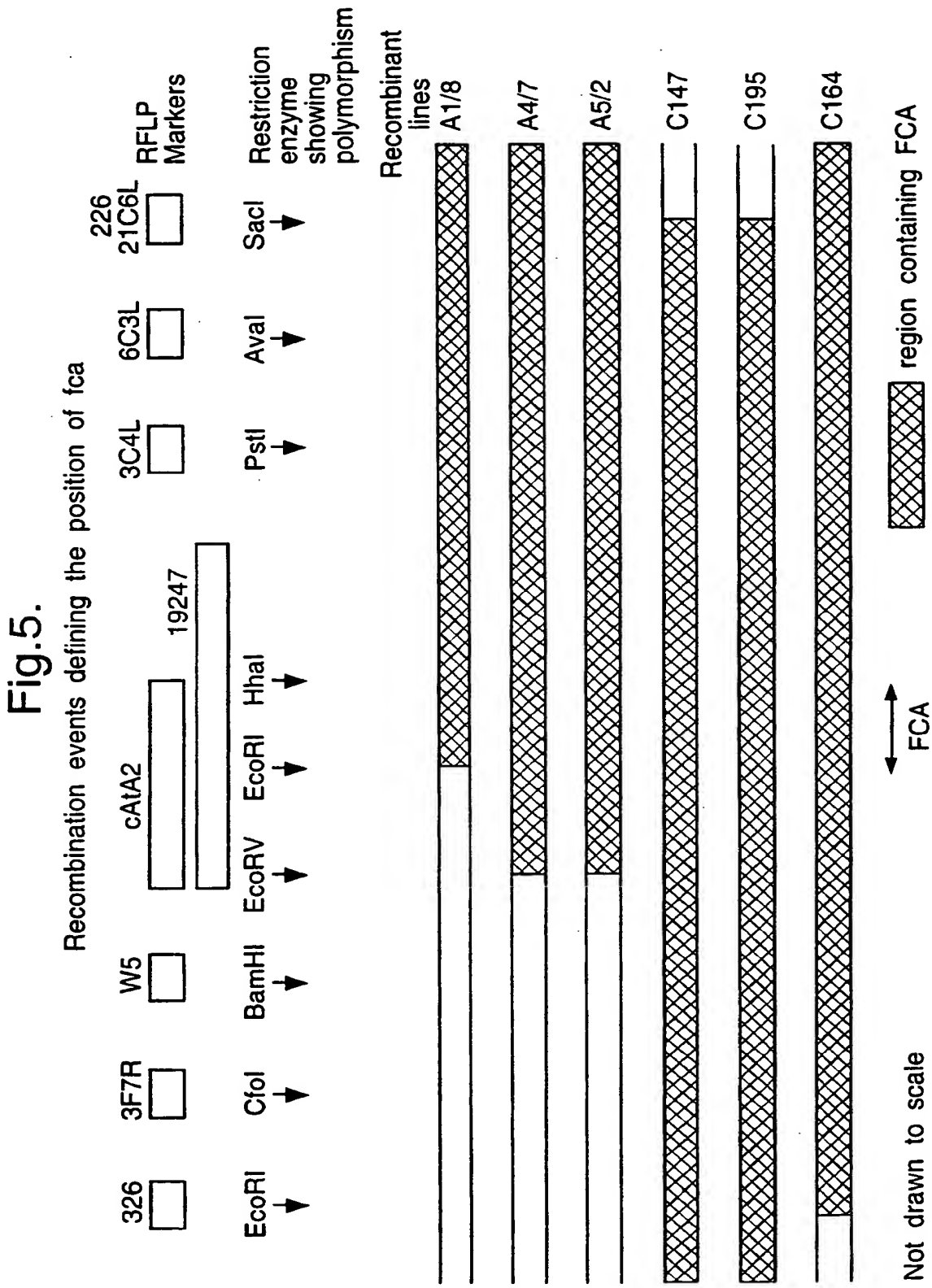
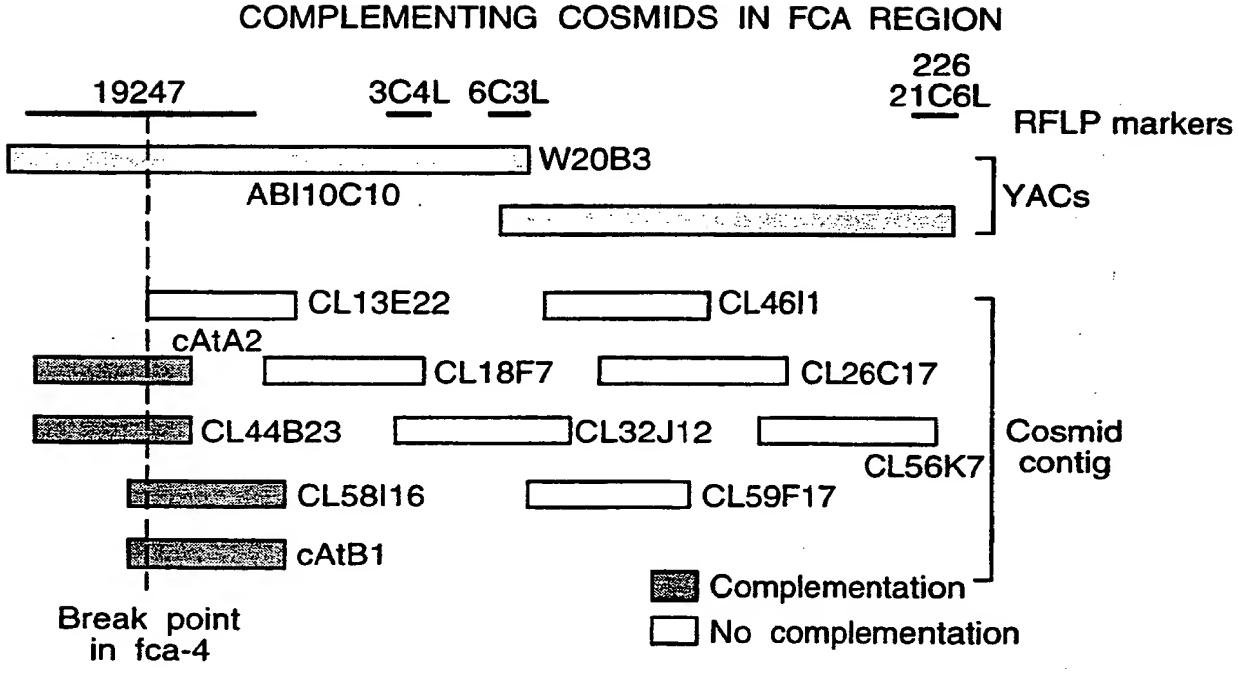


Fig.6.



Cosmid	No. transformants complementing	No. transformants generated
cAtB1	5	5
cAtA2	3	4
CL44B23	7	8
CL58I16	6	11
CL13E22	0	4
CL46I1	0	1
CL32J12	0	4
CL18F7	0	4
CL59F17	0	2
CL26C17	0	2
CL56K7	0	4

Fig.7.

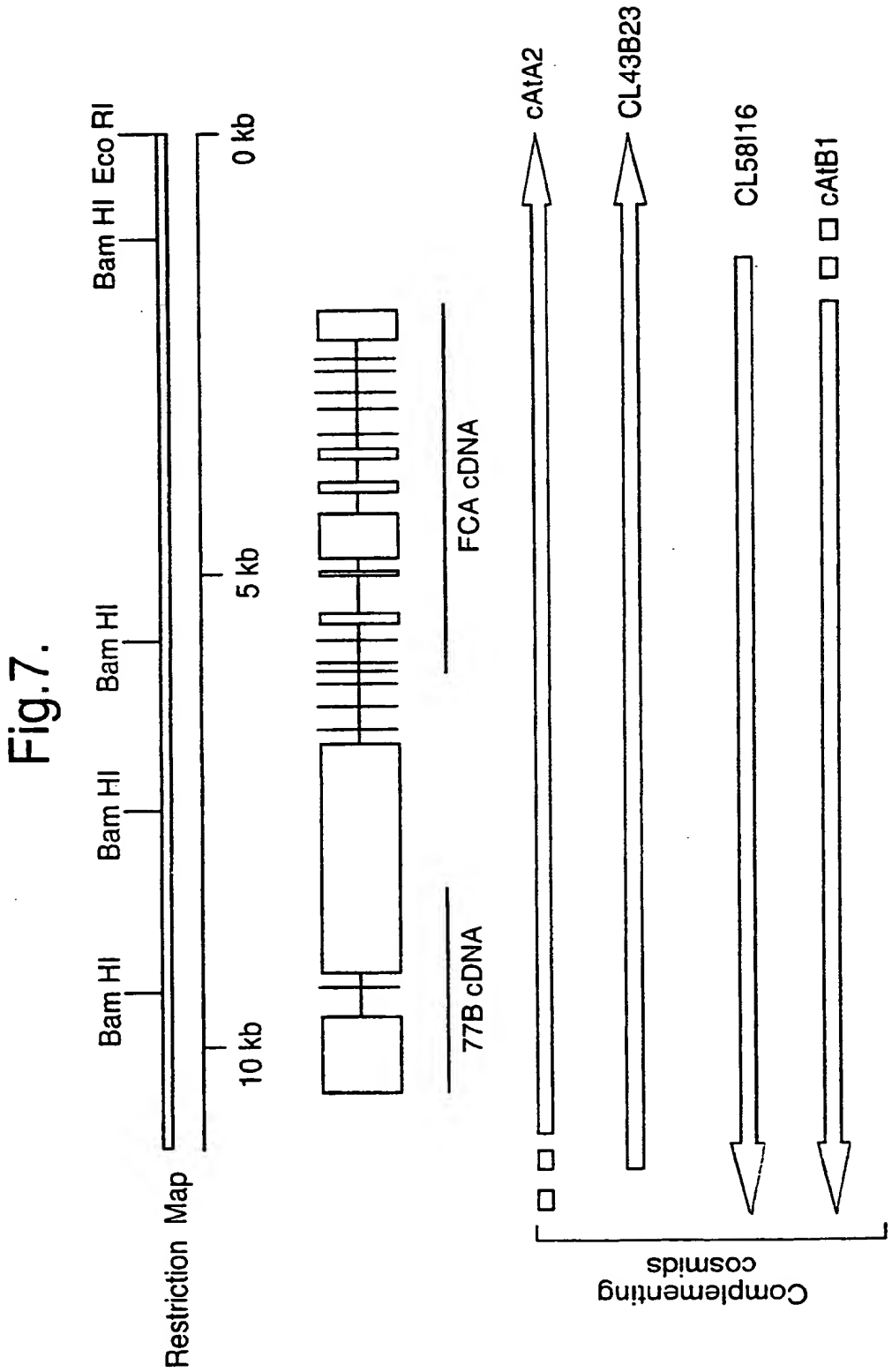


Figure 8a

```
   1  GTTAACGGAT CACACAGTAT AATATAAAAC TAGGTGTTTT GCCCGCACAT
  51  GCGAGCATAA TTTTCTCATC AATACTTATT AGTTTATATC TTATTAATCT
 101  AAAACCAGCA TGATAAGTTA TTATTTATGT TTTCAGATAG TTAAATCAAA
 151  CATCAAAGTA TTTATATATG TCAAATATTT TATCAAAAAT ATATACTTAT
 201  TATTGTGTTA AATTTTTTAA AACACTCATA TCTTAGAAAT AGTTTAGAAA
 251  ATATCTTTAT ATAAATGTTT TTTAACTTTT ATAATAAAAA TATTGTTTTC
 301  AGATAGCAAC AAAATATATA TAGAATTAAC TTATTTTTAA ATTTTTTGAT
 351  AATTTTATTA TATTATTTAA GAATCAATTA TTTATATTAA TATAACATAT
 401  AATTTTCACT GATTAAATAA AATTCGTTTT TAATTATATA AATTCATTAA
 451  GAGTATTGTT TTAATAACAC ATTAGCGAAC ATCAGCTAGA AATTAATAAT
 501  AAATCAATAA CCTAGCTAAA AGTCTAAAAC CTAATAAAAT ATGACAAATA
 551  AGAAAAATTA ACTAAATTTT AATATAAAAT ATAAATTTAA TATTACTAAA
 601  ATAAAATTCA TTTTTAATAT ATATAAGATT CTTAAGGGTA TTTTTTAAAT
 651  TAATAAATTA GTGACTTAGC TAAAAATAAA TAATAAATCA ATGATTTAGC
 701  TAAAACCTAA TAAAAACATG ACAAATAAGC AAATTTACTA AAATTATTGA
 751  TAATATAAAA TATGATTGAT TCTTTAATAC AAAATTAAAA TAAGAGTTTT
 801  TTTAAATCAA ACATAAGTCT GCCGTATCGG TGTTAAAAAA AAAATCATTA
 851  ATAGTGTCGT AGGAATTATG TATTTCCATT AGCGAATAAA ATTGAAGCAG
 901  AGTGTTGGAG GATAGCTCAA CGTATAGGCG AGATTATGGA GATTGATATG
 951  GGAGTTGCCG TAACGGACAC AACTGTTCCT CTGCAAAGAA ACGCTCCTAC
1001  TAGATGGACA TGTCAAGTTG ATGCATCCTG GATAAATGAA AGAAACATAT
1051  CTGGACTTGG CTTTGTGTTA ATGGATGGTG ACTTCCCAAT ACTGTTTGTA
1101  TCAACGGCCG ATATACACGC ACCAAATCAC CACTGCAAGC GGAACGTGAA
1151  GGTTTGCTAT GGGCAATGCA AGAGATACTG AAGTTTGGAC GCAGAGTGAT
1201  GGTCTTTCAA TCGACTATGA ACAACTGGTT ATACTCATTC AAAAGGAGGA
1251  AGATGGCCTG CTTGGACTCG GAGCTCGACG AAATACAAGT TGTATCAAAG
1301  AATTTTCTGA AATTTCTATT GCTTATATTC CTAGATCTTT AAAATTCCGT
1351  ACGAATAGCC TAGCAAAAGG TGTCGATCAC CCGCATCACG ATCAGCTTTT
```

Figure 8a Continued

1401 GTAACCCTTT GCACCAGTGG CTAGCCCACA GCTAGCATGA GGGTGCAAAA

1451 GAGAATAAGT CGAAACAAGC TAGCATGAGG GTGCCAAAAA AGAGAATAAA

1501 GTCGAAAGTA AAACTGAATA TCCAATGAAC AAAATTATCA GAAATCCATA

1551 TTTATGTGGA TGTCTATATG GGACAAACAA TTTTTTTAGA TCAATCCTAA

1601 AATATATAAT TTAAAAAAAC CATTTAAACA AACCATCAAA ATTTTGAATA

1651 TTACACCAAA AAAAAATATA AAGACCAACT ATATTATATT CATGTATAAT

1701 GTGTAGTGGT AAGATTCAAA AAAATTAACT TACTTTACAG TAAGGGAAAA

1751 TTAGATTTTT TATTCCATAT TTACAGTAAA AACATAACAT TTTATAAAAC

1801 TAAACAATTG ACATAATAGT ACAAAATATG AAAAAAAAAT CAAAATACTA

1851 AGAACCTACT ATTAGTTAAA TTAAGTACAG TCAAGTCAAC TAGTATGTGA

1901 ATGAGATTTA ACTTACAAAT TCATTACGAG ACAATAGCAC ATTTAGAAGA

1951 ATAACATGTA GATTGATGTG CACACAAAAA AAAACCAACG GGTACAAATG

2001 TTAACCGCTC CACCGGTCGA ACCATAATCC AGACCGGTTT TGCTATTTAA

2051 ACCGCTCAAA TCGCAAAGTA CGTTTCGCTT ACTTCCAGCA AACCACCATT

2101 GATCTCTCCT CCAATTCACA AATCCAATTT CTCTAGGGTT TGATTTCTTC

2151 GACTTGAATT GCATTTCCAT CCGAATTTCC CCAAATTCGT CAAGCTGGAT

2201 AGGCACCGAG GGGATCGCCA CGAGAGTGCC TTACGACGAT TCCTACCGTA

2251 ACCACCGAGC TCACCTAGAG GTCCCCCTCT ACTCTCAGAT TCACCCTCCA

2301 TGTCACGTTT CGGCGAGGAT GACGAAGGTT TCAGCCGCCG TCGTCGCCGT

2351 GGAAACAGCC TAGCAATTAT CAGTTGGATG GGACAGAGGA GGTGGCGATC

2401 GACGCTGGGA AGATGACGGC CACGATCGTA TTTCACAGAG AGGCGTGGGA

2451 GAGTAGAATT TCAGCCTATG GGTTATGGCT TCGACGGAGG TTTTCCGCCG

2501 ATGAGTCGCG ACGGAGGATT TTGGCCTAAC GTGCCAGTGA ATTTTCCGCC

2551 ATCGGAAAGT CCAGATGCAG GGGGATATTC CGGCGGCAGG GGATTTCAAT

2601 CAACGGGGCC TGCTTACTCT GTGAGATTGA CTTCACCGCC GATCCAGCAG

2651 CCTCTTTCTG GTCAGAAAAG AGGTCGTCCT CTCTCGGAGC AGAGTAGCTT

2701 TACTGGAACT GGTAAGCTTG GGCTCACTCT ACTGTAATCG AGTTGTTTAG

2751 AGTTAACAGT GGTTCATTTT ATACTTGTAT GTGATAATCA GGCTATTTCC

2801 AAACTAAATT ACCTTTACTG GATCATTCGT TTTGCAGATT TACTGATAGT

Figure 8a Continued

2851 AGCAGTATGG TGAAGCTTTT TGTTGGCTCT GTACCAAGGA CAGCTACAGA

2901 AGAAGAAGTG AGTTCATCTT TTTCTTATTT TCCTAATTTC TTCTCAATAT

2951 ATATGCACTT TCTTGAGGCA ATCTAAACCA CGAAGCTCGT AGACTCTGTT

3001 CATAAGCCGT TCTTGTTTAT CATTTTGGTT TTCATAGGTC CGTCCCTTTT

3051 CGAACAACAC GGTAAATGTT CTTGAGGTTG CTTTTATCAA GGACAAGAGA

3101 ACAGGACAGC AGCAAGGTAT GTTTATCTCC ATTTTACTAG GAACAGTCGT

3151 GATTTATGCT TCTAAATTTT TCAGGTCTCC TGAAAAGGCT GATGGGAACG

3201 AACCCCAGTC TCATCATTGG CCTCCATTAG TTTTCAACAA TTTTCGGGCT

3251 TTTGCTTATG CTAGCGAGCG TCTTATCTGT GTTGCTTTGG CACAGAAGAA

3301 GGCTGCCTGT TTAGTTTACT AAGAAAGGGT TTTTGTATTG ACCTTGGTAA

3351 AATAGTTTTT GCGACTTGTG TCCATCCTAG AACCTTAGTT GTGTTTGAAC

3401 AGTGTAGCAG ACTTTATCAT GTTTTAGAGT TGGAGTTAAT GTACATAAAA

3451 TTGAACAGAT GTTTTACTGT TGCCTTTTAG TTGGCACTGG TTTAAAGAAC

3501 GTTGTTTTCT CCTTTCCTAT TGAATTCAGT ATCTCTTTAC TCTTCCTTTC

3551 GATGAATGAA AATGGTGTAT ATGGTCTTGA CTGGATGAAT GTATTTTTAC

3601 TTGGTAGTCT TACAACGTTC ATAAAATGGT TTGATTGATA AACCACCCCT

3651 GCTAGTCAAT ATTTGGCAGT TTCTTAGTGA TTATATCATG TTGGATGTTT

3701 TGTTTCTTTA GTTTCTTTAA TATCAACTTT GGATGTACCG TCTCTATTGG

3751 TTGATGATGA AATTATTTTT TACCATTTTG GATGCTTGAT GCCTTAATGA

3801 ATGGATCTTT CCTTTTTTTC TTATTGTGGA TGGCCGAGGA ACTATAATGA

3851 ATGTTCTCTT CGCTTTTTTT GAATGGCCTG GGATGTGGAC TTCTTGTATG

3901 TTCTCACTTT CATTGATGAA TGACTGTTTC GTTGAGTAGC TCTATTGTTC

3951 TGTATGGTAA CGCTAACACT GCTGATCTAC ATTATGTGGA AGAGATCATA

4001 TGTCTAATGA TATTTTTTTT CTATGTACCT TTCACCAACC AAGCTCAAAA

4051 GCTTGGTTTC AGTTTTTAGT GGTCTTATTC TATATAGAGC TTGGTTTCAG

4101 TTTTTAGTGG TCTTATTCTA TATATTGAGA TTGCTCTTGA AAAATTCCAT

4151 CAAAGTTCTG TCTTTATGAT GCAAGTGTGA AGAATTACTA GATGATGAGT

4201 GATGTATTAT TTAATAATTC GGGACTTTCC AAGAAGTTAT TGTACGGTGA

Figure 8a Continued

4251 CATAAAAGCT TTTACTCATC CCGTTATCAC GGTTTGACTG TAGTAGATTT
4301 GACACATTCC TTGGTTTGAA ATGTTACATG GTGCTAAGAT ATGGAAGGCA
4351 ACGATTATTA TAATTTCTTA GAAATACGTC TTAGCTTTCA CTCGCTCTCA
4401 TTGCTTCGAT CAGCATCAGG CATGAGCCGC CTTAGTATGT ATTTAATGAA
4451 GCAAGTGTCA TTCTTCTCTA TATGCAACTA TTACCAATGA ATTGACGTTG
4501 GGTTGTGGTT ATGTCTCTCA GAACTGTAAT TCTTTTTGTG AATGTCGTCA
4551 AATGTGTGGT GTATGTTGTA TGGTGTATGG TGACGAAAAT GTGATGTATG
4601 GCTCTAGTTT TAATTATATC ATTTGTTACT TAGCAGTGAT TGAGAACTCT
4651 TAACTTGTAA TTTTATCTAA TTTTTTTTTG CAGTGATTGG ATTCTTTTTG
4701 CGTAATATAT ATTTTATTTG CAAATACCGA CTGTGTTCTT TTTAAATAGT
4751 TTAAAGGCAT ATGCTTTATT TGAAGCACAT TAGTTTATTA TTCTCTCCAT
4801 CAAATCTACT ACAGTAATGT AAGTCGAGGC TGTCAGGACA TGTCTTATGA
4851 TTTTCGTACT GAAACTTATG TGCTTTCAAT GTGGTCGTGG CTTGTACATT
4901 TGTAAAGAAA CTATTTACTA GTATCTCTTG ATGTTTGATG GAGGGACAAG
4951 TGGAACCTTG AACAGAAGCT TATGTAGCAG TCTTTAATGC AGGCTGTTGT
5001 TTTGTAAAAT ATGCAACTTC TGAAGATGCG GATAGGGCCA TTAGAGCATT
5051 GCACAATCAG ATCACTCTTC CTGGGGTAAC TACCATTGAT GCCTTCTCTT
5101 ATCAAGGACA GGAAAATACA GGTTAACTCT ATCTTTACAA TTTGCTGATT
5151 CCCAGGGAAC TGGCCTTGTT CAAGTTCGAT ATGCTGATGG GGAGAGAGAA
5201 CGCATAGGTA ATCAACTTTC GCGCCATATT ATCTGAATCT GGCCTTCATT
5251 GTCTGGTATA CATAGGGTGA CCATACGCTG TACAAATTCA AATTACGAGA
5301 ATTGAGATAA TGTGGGAAAC TATATGAATC TTAAGGAAGT GGATCCTTTT
5351 TTCTGTGGTC CTTGCCTCAC TCTCAAGTAT TAACTGATTG AATTTACTTC
5401 TTCTGAAGGT GCGGTAGAGT TTAAGCTTTT TGTTGGTTCC TTAAACAAGC
5451 AAGCCACTGA AAACGAGGTT GAGGAGGTAT GTCTCATATC CTACTTTTTG
5501 ATGGAAAGTA ATTACTTATG TCTGATTTAC AAAGAGGGAA GCGTTCTAAA
5551 TTTAGATATT ACAGTATCCC CTGTCGCCTT AGCTGGTAAT TTTAGTGATT
5601 ATATGACAAT TTAGTAGTCC TCTTGGAAGG GTCAGCGGCT TGAAATTTTG
5651 TGTCAACTAT TCGAGCGCTT ACACATTTTA CTAACTGAGT GATCTCTTCT

Figure 8a Continued

5701 TTCAAATGGA CTGACTGAGT GATCTCTTCT TCCAAATGGA TGTAACTTTT

5751 TGGCTGTCAG CTTTCTTTTC TCAGTAAATA TGATGAAGAT GTGAACGGCT

5801 ACTTTGTCCT GTTGTTGCTT TAACAGCTCT TTTTGCAATT TGGTCGCGTG

5851 GAGGATGTCT ATCTCATGCG TGATGAATAT AGACAGAGTC GTGGTATGTC

5901 TGGTAACTGC CACTAGACTC TATAACTCGT TTGATGGTGT TGATATGGTC

5951 AAACTGTTTT TGACACTCAT TTAGGATGCG GGTTTGTTAA ATATTCAAGC

6001 AAAGAGACGG CCATGGCAGC TATCGATGGT CTCAATGGAA CTTATACCAT

6051 GAGAGTAAGC TGTGAAATCA CATGAGTATC TCACTTTCTC TCATTATCCC

6101 CTCTAGACCT GTTTTGTTTA CTGGCCTCTT TCCCTTCTCC AGGGTTGCAA

6151 TCAGCCATTG ATTGTTCGGT TTGCTGATCC AAAGAGGCCT AAACCGGGCG

6201 AGTCAAGGTA TTGCCTTGGA GACTATATTT TGAATTCATT ATAATGCTAA

6251 TATCAAAAAA ATTGTGTCTA CTGTCATTGT TTGTTCTATT GAGTTACATT

6301 TATGAGAATC TTTTGGGGCA TGGGTGGAGG AGAGCTGCGA ACCTTATTCC

6351 TTCTCCAGTT ATTACTTGAA TGCGATGAAT TTCTTTCTAT ATATCCTTAA

6401 CTAGTTTCTG TTTCCAGGGA AGTGGCACAT CCTGTTGGAC TTTGTTCAGG

6451 GCCTCGTTTT CAAGCTTCAG GACCAAGGTG ACTGGGGTGA AAGGAGATCG

6501 TTGTTTTTGT CATCAATTAA TTATATATTT TGACTAAACG TGGTCTCCTT

6551 ATCTTCATTT GTTAGGCCTA CATCTAACCT TGGTGACCTT AGTGTGGATG

6601 TGAGCCACAC AAATCCTTGG CGTCCTATGA ATTCACCAAA CATGGGGCCA

6651 CCTGGTAACA CTGGGATCCG TGGTACCGGA AGTGACTTGG CTCCTAGGCC

6701 AGGTCAAGCC ACATTACCTT CAAATCAGGT AAGAACAGCT TGATGATCAT

6751 GTATATTATC TTATATGTAC ACACCCAATC ACACATAAAG TAATCGGGCA

6801 TAAGGTTTTA CATGTATTGT GTGAGTAGGA CGAACATAAT TTATATGCTG

6851 CACATATATG AGCGTATGGA CTCTTGAAAA GAAGCATGAA GTTCCGACCT

6901 TCCAGCTTTT CATATGATGC AGCAAACTTG ATGTGTTTTG CATTGAAATG

6951 ATATGGCTTT GATTTGCATT TTGTCAGTTT CTAAGGAGTT TTTTTCTTCA

7001 ATAATTTCTA CTTCTGATGT TAGCTTTATT TGTGGCATTC TATAATGTTA

7051 GGGAGGTCCA TTGGGTGGTT ATGTTGTTCC TGCCATTAAC CCTCTACCAG

7101 TCTCATCCTC TGCCACATCG CAACAGGTAC TTCAGCTGAA TTTTTCCAAT

Figure 8a Continued

7151 AAAGAAAATC TGAAAATGTT GTGTTGATCA GTTAATTTCA ACTGTTTCTA

7201 TTCCATAGCA AAACCGGGGA GCTGGCCAGC ATATGTCACC ATTACAAAAA

7251 CCTCTTCACA GTCCACAGGA TGTGCCCCTT CGACCACAAA CTAATTTCCC

7301 TGGGGCCCAA GCATCCTTGC AGAATCCTTA TGGTTATAGC AGCCAGTTGC

7351 CTACTTCTCA GCTGCGGCCA CAACAAAACG TCACTCCTGC AACAGCTCCT

7401 CAAGCTCCTT TGAACATCAA CCTACGGCCA ACACCTGTAT CTTCTGCAAC

7451 TGATCAATTG CGCCCTCGTG CTCAGCAGCC ACCGCCACAA AAGATGCAAC

7501 ATCCTCCTTC TGAGCTAGTT CAGCTCTTGT CACAACAGAC TCAGACTCTA

7551 CAAGCAACCT TCCAATCATC TCAGCAAGCA TTTTCTCAAC TGCAGGAGCA

7601 GGTGCAGTCC ATGCAGCAAC CAAACCAAAA ATTACCAGGC TCACAGACTG

7651 GCCATGGTAA ACAGCAGGTA CAAACATAGT TCCCTGTTGC ATCTGTCCAG

7701 TCCAGTTCCT CAGCTGTTTT TGTTGTTTTA ACTTACAATT ATTTCCTGAT

7751 GTCTAAGTAT TCAATCCTTC ATATATTTTA GTAGTCCCTC TTTTTTATTA

7801 TGTTTTTCTC GGTTGCTTCT CTATCAGTGG GCTGGATCTG CAATTCCGAC

7851 AGTTGTTAGC ACCACTGCTT CTACACCAGT TAGCTATATG CAAACAGCTG

7901 CACCTGCAGC AACTCAGAGT GTTGTTTCTC GCAAATGTAA CTGGACCGAG

7951 CATACCTCGC CTGATGGATT TAAGTATTAT TACAACGGTC AAACCGGTGA

8001 AAGCAAGGTG AGAAACGTGG TTCCTCTTTA GTTATGTTCT CTTGTGAGTT

8051 TCAGGAGGAT TCCTTGTATT TGCTGTGCTA TTTATTATCC TTGAACATGT

8101 ATATGTATAG ATTTCATATT TGAAGTTCAT CAATACGTGT CGTAATATAA

8151 TTGACTTTTG CAGTGGGAAA AACCTGAGGA AATGGTATTG TTCGAACGTC

8201 AGCAACAGCA GCCAACTATA AATCAGCCCC AGACCCAATC ACAGCAGGCT

8251 CTTTATTCCC AGCCGATGCA GCAACAACCA CAACAGGTTC ACCAGCAATA

8301 TCAGGGCCAA TATGTACAGC AGCCTATTTA TTCTTCAGTG GTTGGTTCTG

8351 TTTTCTTGCT GCTTACATCC ATATAGTTTT CTCACATGGT CTCTAACTTG

8401 AATATGTATT CTTTTCCATT TGGAGTTGCA GTATCCAACT CCAGGGGTCA

8451 GCCAGAATGC TCAGGTGTAT ATTTAGTTAA ATTATTTGCT TATCTTTCAT

8501 TTCAGAATTT GATCATTGAG TTACCAATCT AGTGGGTATA AGGAGACGGG

8551 CCACTTTATG CAATAAACCA TGGTTTTACA AGCGTTTTGA ATATACAGAT

Figure 8a Continued

8601 ACAAACATCT AAATTTGATC ATTTCAAAAT TTGATCATCG AGTTCCCTTT
8651 TCAATGCAGT ATCCGCCGCC ATTGGGAGTT AGTCAAAATA GCCAGGTACA
8701 TATTTGAACC TTATTTCACG TGGGACTAAT TGAAATTCGA TTTTGATATG
8751 CATTTCATAA ATGTGAAGAT TTGATGAGTG GTCGTTTTGG TAGCGTTTTA
8801 GAAAACGATA TGCATATATT CTCTAGTTGA ATATTTCTTT TTTCTGGAAC
8851 ATGCAGTTTC CTATGTCAGG CACCGGTCAA AATGCTCAGG TACATATTAT
8901 ATCATGCATC ATATCTTCTG ATTAACTTCA AATTTAATCA GAAAACATAC
8951 GTGAGATTCC AGTAGAAACA AAATCGATAT GACTGTATTG GTTGGAAATG
9001 GTTAAGGCAG AGCTCATGTT CTAATGTGTT AAAATTTTCT AGGAATTT

Figure 8b

```
  1  MGYGFDGGFP PMSRDGGFWP NVPVNFPPSE SPDAGGYSGG RGFQSTGPAY
 51  SVRLTSPPIQ QPLSGQKRGR PLSEQSSFTG TGLLIVAVMV KLFVGSVPRT
101  ATEEEVRPFS NNTVNVLEVA FIKDKRTGQQ QGCCFVKYAT SEDADRAIRA
151  LHNQITLPGG TGLVQVRYAD GERERIGAVE FKLFVGSLNK QATENEVEEL
201  FLQFGRVEDV YLMRDEYRQS RGCGFVKYSS KETAMAAIDG LNGTYTMRGC
251  NQPLIVRFAD PKRPKPGESK EVAHPVGLCS GPRFQASGPK PTSNLGDLSV
301  DVSHTNPWRP MNSPNMGPPG NTGIRGTGSD LAPRPGQATL PSNQGGPLGG
351  YVVPAINPLP VSSSATSQQQ NRGAGQHMSP LQKPLHSPQD VPLRPQTNFP
401  GAQASLQNPY GYSSQLPTSQ LRPQQNVTPA TAPQAPLNIN LRPTPVSSAT
451  DQLRPRAQQP PPQKMQHPPS ELVQLLSQQT QTLQATFQSS QQAFSQLQEQ
501  VQSMQQPNQK LPGSQTGHGK QQWAGSAIPT VVSTTASTPV SYMQTAAPAA
551  TQSVVSRKCN WTEHTSPDGF KYYYNGQTGE SKWEKPEEMV LFERQQQQPT
601  INQPQTQSQQ ALYSQPMQQQ PQQVHQQYQG QYVQQPIYSS VYPTPGVSQN
651  AQYPPPLGVS QNSQFPMSGT GQNAQ
```

Figure 9

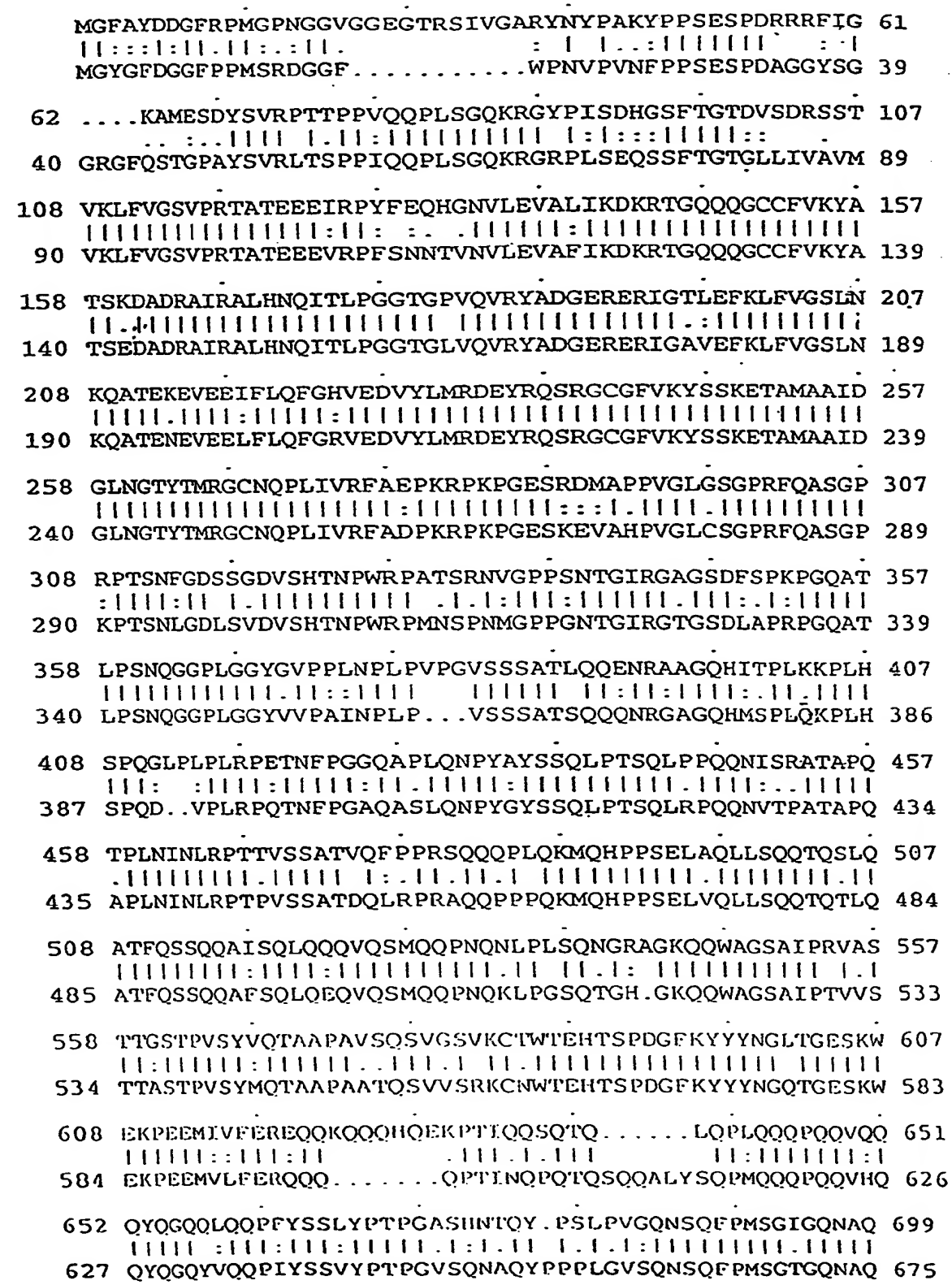
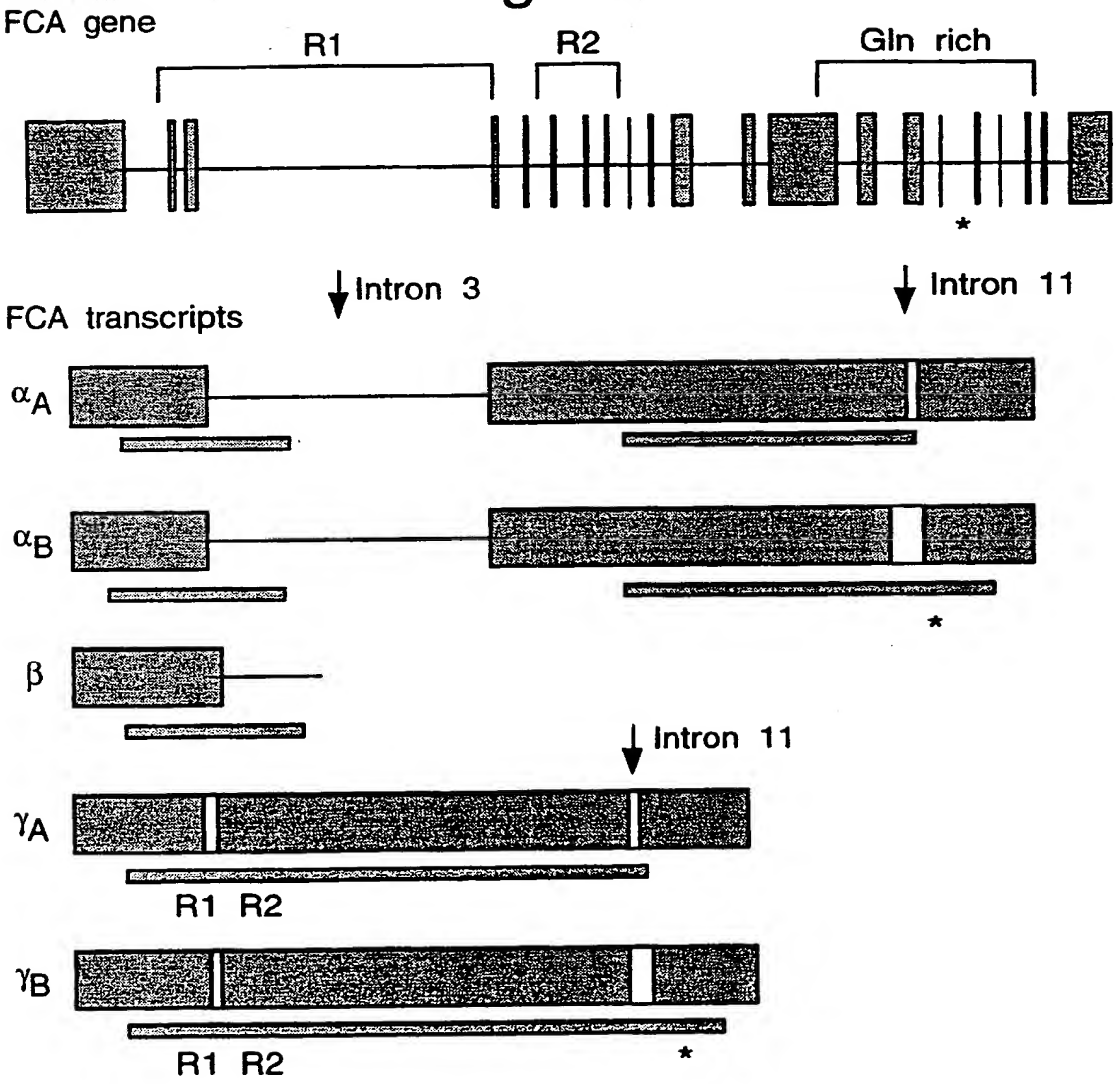


Fig.10.



This Page Blank (uspto)

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/29, 15/82, C07K 14/415, A01H 5/00 — **A3**

(11) International Publication Number: **WO 96/38560**

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/GB96/01332

(22) International Filing Date: 3 June 1996 (03.06.96)

(30) Priority Data:
9511196.9 2 June 1995 (02.06.95) GB

(71) Applicant *(for all designated States except US)*: JOHN INNES CENTRE INNOVATIONS LIMITED [GB/GB]; Norwich Research Park, Colney Lane, Norwich NR4 7UH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DEAN, Caroline [GB/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB). MACKNIGHT, Richard, Colin [NZ/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB). BANCROFT, Ian [GB/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB). LISTER, Clare, Katharine [GB/GB]; John Innes Centre, Molecular Genetics Dept., Norwich Research Park, Colney, Norwich NR4 7UH (GB).

(74) Agents: WALTON, Seán, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
9 January 1997 (09.01.97)

(54) Title: GENETIC CONTROL OF FLOWERING

(57) Abstract

FCA genes of *Arabidopsis thaliana* and *Brassica napus* are provided, enabling flowering characteristics, particularly timing of flowering, to be influenced in transgenic plants. Timing of flowering may be delayed or hastened using sense and antisense expression, also various mutants and alleles, including alternatively spliced forms.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

INTERNATIONAL SEARCH REPORT

Inter··onal Application No
PC1/GB 96/01332

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 C07K14/415 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANNUAL MEETING OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY, BIRMINGHAM, ALABAMA, USA, APRIL 7-12, 1991. J EXP BOT 42 (238 SUPPL.). 1991. 48., XP002018457 BANCROFT I ET AL: "THE DEVELOPMENT OF SYSTEMS FOR THE ISOLATION OF GENES FROM ARABIDOPSIS-THALIANA BY CHROMOSOME WALKING IN YAC LIBRARIES TOWARDS THE ISOLATION OF THE FLORAL INDUCTION GENE FCA." see abstract P8.62 --- -/--	1-14,28, 29, 31-37,39

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 1996	29. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	JOURNAL OF EXPERIMENTAL BOTANY, vol. 45, no. 278, September 1994, pages 1279-1288, XP000609516 CHANDLER, J., ET AL.: "Factors influencing the vernalization response and flowering time of late flowering mutants of Arabidopsis thaliana (L.) Heynh." see page 1288, right-hand column & THIRD INTERNATIONAL CONGRESS OF PLANT MOLECULAR BIOLOGY: MOLECULAR BIOLOGY OF PLANT GROWTH AND DEVELOPMENT. TUCSON, ARIZONA. ABSTRACT NO. 508, 1991, WESTPHAL, L., ET AL.: "Cloning FCA, a late-flowering locus of Arabidopsis thaliana (L.) Heynh." ---	1-14,28, 29, 31-37,39
X	EMBL SEQUENCE DATABASE, REL.42, 31-JAN-19995, ACCESSION NO. T42029, XP002018288 NEWMAN, T., ET AL.: "5292 Arabidopsis thaliana cDNA clone 110C13T7" see sequence ---	1-4, 28-30
P,X	SEMIN. CELL DEV. BIOL. (1996), 7(3), 435-440 , XP000609514 WILSON, ALLISON ET AL: "Analysis of the molecular basis of vernalization in Arabidopsis thaliana" see page 436, right-hand column - page 437, left-hand column ---	1-4, 8-10,29, 30
O,P, X	ANNUAL MEETING OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY: PLANT BIOLOGY ABSTRACTS, LANCASTER, ENGLAND, UK, MARCH 24-29, 1996. JOURNAL OF EXPERIMENTAL BOTANY 47 (SUPPL.). 1996. 7. , XP000609515 MACKNIGHT R ET AL: "Characterisation of the Arabidopsis FCA gene: Required for the early transition to flowering." see abstract CP2.3 ---	1-4, 8-10,29, 30
A	MOLECULAR AND GENERAL GENETICS, vol. 229, 1991, pages 57-66, XP002018289 KOORNNEEF, M., ET AL.: "A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana" cited in the application see the whole document --- -/--	1-40

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE PLANT CELL, vol. 6, no. 1, January 1994, pages 75-83, XP002018290 LEE, I., ET AL.: "Isolation of LUMINIDEPENDENS: a gene involved in the control of flowering time in Arabidopis" cited in the application see the whole document	1-40
A	CELL, vol. 80, 24 March 1995, pages 847-857, XP002004926 PUTTERILL J ET AL: "THE CONSTANS GENE OF ARABIDOPSIS PROMOTES FLOWERING AND ENCODES A PROTEIN SHOWING SIMILARITIES TO ZINC FINGER TRANSCRIPTION FACTORS" see the whole document	1-40
A	DATABASE WPI Section Ch, Week 9243 Derwent Publications Ltd., London, GB; Class C06, AN 92-354683 XP002018484 & JP,A,04 258 292 (JAPAN TOBACCO INC) , 14 September 1992 see abstract	1-40
A	PLANT MOLECULAR BIOLOGY, vol. 25, 1994, pages 335-337, XP002019105 AN, G., ET AL.: "Regulatory genes controlling flowering time or floral organ development" see page 335, left-hand column	25,26
A	MOLECULAR AND GENERAL GENETICS, vol. 226, 1991, pages 484-490, XP002018458 GRILL, E., ET AL.: "Construction and characterization of a yeast artificial chromosome library of Arabidopsis which is suitable for chromosome walking"	
A	MOLECULAR AND GENERAL GENETICS, vol. 239, 1993, pages 145-157, XP002018459 PUTTERILL, J., ET AL.: "Chromosome walking with YAC clones in Arabidopsis: isolation of 1700 kb of contiguous DNA on chromosome 5, including a 300 kb region containing the flowering-time gene CO" see the whole document	

3